HERPES SIMPLEX VIRUS-1 (HSV-1) AS A GENE DELIVERY VECTOR FOR NEURAL PRECURSOR CELLS

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ABSTRACT

The thesis work presented evaluated the potential for use of disabled herpes simplex virus-1 (HSV-1) as gene delivery vectors for neural precursor cells and studied the effects of delivered recombinant factors on the *de novo* development of dopaminergic neurons from neural precursor cells.

Highly and less disabled HSV-1 has been studied with respect to gene delivery efficiency and effects on cellular integrity in primary neural progenitor cells, neural stem cells grown as neurospheres, and in endogenous neural stem cell niches in the adult rat. Data from autografts of virally transduced neurospheres into the striatum of rats were also presented. The characteristics of virally transduced neural precursor cells were compared to other viral vector systems reported in literature.

With respect to the study of differentiation factors, work has concentrated on fibroblast growth factor 8b (FGF8b). This has demonstrated that FGF8b is a mitogen for neural precursor cells *in vitro*. The study showed that neural stem cells isolated from different regions of the developing brain can be expanded in FGF8b alone and retain their stem cell characteristics, e.g. the capacity of self-renewal and multipotentiality. Growth curves and dose responses of neural precursor cells expanded in FGF8b further confirmed these findings. The study also showed survival effects of FGF8b on dopaminergic neurons derived from mesencephalic precursor cells. Further the effects of FGF8b on proliferation and differentiation of endogenous stem cells were also investigated.

Finally, the thesis work involved the construction and generation of highly and less disabled viruses expressing FGF8b, sonic hedgehog, basic fibroblast growth factor, and the transcription factor nurr1. Expression and bioactivity of the various constructs was confirmed. The effects of these factors on dopamine neuron development were then studied *in vitro* using neural progenitors and neural precursor cells for which gene delivery had been optimized in the first part of this thesis.

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DECLARATION

I hereby declare that my thesis is not substantially the same as any that I have submitted for a degree or diploma or other qualification at any other University. I further state no part of my thesis has already been or is being concurrently submitted for any such degree, diploma or other qualification.

The work presented in this thesis is entirely my own. The vector RL1+/27+/4-pR19Nurr1wpre was generated by J. Palmer (BioVex) using the Nurr1 encoding plasmid constructed by myself. The construct 1764/27-/4-pR19GDNF was kindly provided by X-P. Li (BioVex).

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
A	adenosine
AA	ascorbic acid
AADC	aromatic L-amino acid decarboxylase
AAV	adeno-associated virus
Ad	adenovirus
ALS	amyotrophic lateral sclerosis
AP	alkaline phosphatase
apaf	apoptotic protease-activating factor
APS	ammonium persulphate
AraC	cytosine-b-D-arabinofuranoside
ART	artemin
BAC	bacterial artificial chromosome
BDNF	brain derived neurotrophic factor
bFGF	basic fibroblast growth factor (synonym to FGF2)
BHK	baby hamster kidney cells
bis	n,n'-methylene-bis-acrylamide
BMP	bone morphogenic protein
bp	base pair
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
C	cytosine, caudal
cDNA	complementary DNA
CIAP	calf intestinal alkaline phosphatase
CM	conditioned media
CMC	carboxymethylcellulose
CMV	cytomegalovirus
CNS	central nervous system
CPE	cytopathic effect
CTX	cortex
d	deoxy

D	dorsal
DA	dopamine
DAPI	4', 6-Diamidino-2-phenyindole
DARP-32	dopamine and cAMP-regulated phosphoprotein of 32 kDa
DAT	dopamine transporter
DBS	deep brain stimulation
dd	dideoxy
ddH_20	double distilled water
DG	dentate gyrus
DIV	days *in vitro*
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
ds	double stranded
DTT	Dithiotheitrol
E	embryonic (gestation day) or early (class of genes)
ECL	enhanced chemiluminescence
EDTA	ethylendiaminetetra-acetic acid
EGF	endothelial growth factor
EGFP	enhanced GFP
En	engrailed
ER	endoplasmic reticulum
ES	embryonic stem cell
EtOH	ethanol
FCS	fetal calf serum
FGF	fibroblast growth factor
FGF8	fibroblast growth factor 8 (accordingly for other growth factors)
FGFR	fibroblast growth factor receptor
FGM	full growth medium
FW	forward
g	gram
G	guanosine
GABA	gamma aminobutyric acid

GAD	glutamic acid decarboxylase
GalC	galactocerebroside C
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gB	glycoprotein B (accordingly for other glycoproteins)
GCL	granule cell layer
GDNF	glial derived neurotrophic factor
GF	growth factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GPi	globus pallidus interna
HBSS	Hank's balanced salt solution
HCF	host cell factor
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
HIV	human immunodeficiency virus
HMBA	hexamethylene bisacetamide
hr	hour
HRP	horseradish peroxidase
HS	heparan sulphate
HSV	herpes simplex virus
i.c.	intra cutaneous
i.p.	intra peritoneal
ICP	infected cell polypeptide
IE	immediate early (class of gene)
Ig	immunoglobulin
IRES	internal ribosome entry site
IRL	internal repeat long
IRS	internal repeat short
ITR	inverted terminal repeat
kb	kilobase
L	late (class of gene), lateral
l	litre
LacZ	beta galactosidase
LAP	latency active promoter

LAT	latency associated transcript
LB	Luria Bertani medium
LTR	long terminal repeat
LV	lateral ventricle
M	medial, molar
m.o.i.	multiplicity of infection
mA	milliamps
MAP	microtubule-associated protein
MESV	murine embryonic stem cell virus
mg	milligram
MHC	major histocompability complex
min	minute
MLV	murine leukaemia virus
mM	millimolar
MMLV	moloney murine leukaemia virus
MPP	1-methyl-4-phenylpyridinium
MPTP	*n*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
MS	multiple sclerosis
MSC	mesenchymal stem cell
MW	molecular weight
NAIP	neuronal apoptosis protein
NeuN	neuronal nuclei
NF	neurofilament
ng	nanogram
NGS	normal goat serum
NIH	National Institute of Health
nm	nanometer
NP40	nonidet P40
NPC	neural precursor cell
NRT	neurturin
NS	neurosphere
NSC	neural stem cell

NSE	neuron specific enolase
nt	nucleotide
Nurr1	Nur-related factor 1
O4	oligodendrocyte marker 4
OB	olfactory bulb
OD	optical density
OI	osteogenesis imperfecta
ORF	open reading frame
p	plasmid
pA	polyadenylation signal
PAGE	polyacrylamide gel electrophoresis
PB	phosphate buffer
PBS	phosphate buffer saline
PBST	PBS with 0.1% tween-20
PCR	polymerase chain reaction
PD	Parkinson's disease
PDGF	platelet-derived growth factor
PET	positron emission tomography
PFA	paraformaldehyde
pfu	plaque forming unit
PKA	protein kinase-A
PLL	poly-L-lysine
PNS	peripheral nervous system
Poly-HEMA	Poly (2-hydroxyethyl methacrylate)
PSP	persephin
Ptc	patched
R	rostral
RMS	rostral migratory stream
RNA	ribonucleic acid
ROS	reactive species
rpm	revolutions per minute
RSV	rous sarcoma virus
RT	room temperature

RT-PCR	reverse transcriptase polymerase chain reaction
RV	reverse
SCID	severe combined immunodeficiency
SCNT	somatic cell nuclear transfer
SDIA	stromal derived inducing activity
SDS	sodium dodecyl sulphate
sec	second
SEM	standard error of the means
SFM	serum free medium
SGZ	subgranular zone
Shh	sonic hedgehog
ShhN	aminotermianl sonic hedgehog
Smo	smoothened
SN	substantia nigra
ss	single stranded
STN	subthalamic nucleus
STR	striatum
SVZ	subventricular zone
T	thymidine
TAE	Tris-actetae EDTA buffer
TAP	transporter associated with antigen processing
TEMED	N,N,N,N'-tetramethylethylenediamine
TGFα	transforming growth factor alpha
TH	tyrosine hydroxylase
TK	thymidine kinase
TNF	tumor necrosis factor
Tris	tris(hydroxyl)aminomethane
TRL	terminal repeat long
TRS	terminal repeat short
TuJ1	tubulin-β-tubulin isotype III
Tween-20	polyoxyethylene-sorbitan monolaurate
UL	unique long
UPDRS	unified Parkinson's disease rating scale

US	unique short
UV	ultraviolet
V	ventral, volt
v/v	volume for volume
vhs	virion host shut off
VM	ventral mesecephalon
VMAT	vesicular monoamine transporter
VP	virion protein
vSNc	ventral substantia nigra compacta
VSV	vesicular stomatitis virus
VTA	ventral tegmental area
w/o	without
w/v	weight for volume
wpre	woodchuck posttranscriptional regulatory element
WT	wild type
x-Gal	4-chloro, 5-bromo, 3-indolyl-β-galactosidase
μg	microgram
μl	microlitre

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INTRODUCTION

1.1 Hype and hope of stem cells

"If DNA is the software, than stem cells are its hardware." [Stephen Hawking, 2004]

On an almost daily basis we are bombarded with public and scientific news released about stem cells. Most recently the opening of the world's first stem cell bank in Hertfordshire (Pincock, 2004) and the construction of a pluripotent embryonic stem cell line from a cloned human blastocyst (Hwang et al., 2004) have caused quite a sensation. Stem cells not only raise the interest of scientists but also politicians, philosophers, lawyers, economists, human right activists and clergy. Often stem cells are mentioned in the same sentence with "curing" diseases, as replacements for worn out body parts, or for the cloning of humans reminiscent of Aldous Huxley's "Brave new world". In any discussion of the potential use of stem cells it is important to evaluate the realistic scientific and therapeutic potential, as well as the involved ethical concerns and to distinguish between the 'hype' and the 'hope'.

Human stem cells exist from the very beginning to the end of life. During this time stem cells are unique as they have the capacity for unlimited self-renewal along with the ability to produce multiple different types of terminally differentiated descendants. The fertilized egg is a totipotent (Latin *totus*, meaning entire) stem cell and generates all the cells of a human. As development proceeds, stem cells become channelled into distinct pathways of differentiation. During a restricted period in development pluripotent (Latin *plures*, meaning several) embryonic stem (ES) cells exist and can be isolated from the inner cell mass of the embryonic blastula comprising a small group of cells (13 at the 64 cell stage embryo) (Hynes and Rosenthal, 2000). ES cells also exist in humans and it has become possible to grow them *in vitro* (Thomson et al., 1998). Development continues and cells become more specialised to give rise to a limited number of cell types, e.g. neuroepithelial stem cells generate all neural cell types, producing neurons first, and glia at later stages (reviewed by Kintner, 2002). The existence of stem cells is not limited to embryonic stages, but also occurs in adult tissue. Mammals appear to possess about 20 major types of somatic stem cells, e.g. in liver, pancreas, bone, cartilage, muscle or blood (reviewed by McKay, 2000). Most

fascinatingly stem cells even persist in regions of the body that have historically been thought of as unable to give rise to new cells or to have a capacity for self-renewal, such as the adult mammalian central nervous system (CNS) (see below).

Reports suggesting that stem cells are not restricted to the generation of cell types specific to the tissue of origin have caused great excitement. This phenomenon, termed "transdifferentiation" (Anderson et al., 2001), proposes that adult stem cells retain sufficient plasticity to be able to cross lineage boundaries. Thus, for example, brain derived stem cells can give rise to blood and muscle cells, muscle derived stem cells to blood, blood derived stem cells to neurons etc. (Bjorklund and Svendsen, 2001; Paul et al., 2002). The method of analysis used and the interpretation of the results obtained have often been controversial (Weissman et al., 2001; Anderson et al., 2001; Bjorklund and Svendsen, 2001; Wurmser and Gage, 2002), and some of the results of the earlier experiments have been later proved to result from cell fusion rather than transdifferentiation (Ying et al., 2002; Terada et al., 2002; Medvinsky and Smith, 2003).

A plethora of clinical applications have been proposed for the use of stem cells including for the treatment of leukaemia, diabetes, spinal cord injuries and ischemic heart diseases (reviewed by McKay, 2000; Paul et al., 2002). However, most prominent is the potential use of stem cells for the development of novel therapies to treat neurodegenerative diseases, such as Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS) or stroke (reviewed by Shihabuddin et al., 1999; Snyder et al., 2004). ES cells particularly have been credited with promise for therapeutic use. For example a report from the National Institutes of Health (NIH; Bethesda) has stated: "This class of human cell holds the promise of being able to repair or replace cells or tissues that are damaged or destroyed by many of our most devastating diseases and disabilities" (NIH, July 2001; http://www.nih.gov/news/stemcell/scireport.htm). However, the current state of scientific and clinical knowledge means that the controlled generation of specific cell types from ES cells and proof of therapeutic function is still in its infancy. Although, for example, insulin releasing cells have been generated from ES cells (Lumelsky et al., 2001; Assady et al., 2001), such insulin production is low, and glucose

responsiveness to the insulin produced has not been well characterized. Another example of the potential use of stem cells is the application to demyelination disorders. Here ES cell-derived oligodendrocyte precursors have been shown to myelinate axons in the brain and spinal cord after transplantation into a rat model of myelin disease (Brustle et al., 1999), and adult neurospheres (see later) injected intravenously or intracerebroventricularly into a mouse model of multiple sclerosis were shown to induce remyelination and functional recovery (Pluchino et al., 2003). However, clinical applications of such findings in human disease are not straightforward since demyelinating diseases such as MS are multifocal. It is therefore difficult to develop surgical procedures to target the multiple sites to which delivery of the cells would be required (Paul et al., 2002). Both of the examples also demonstrated another possible pitfall of the use of stem cells. As both juvenile diabetes and MS are possibly autoimmune diseases (Atkinson and Eisenbarth, 2001; Davidson and Diamond, 2001), rejection of transplanted and differentiated cells could prove problematic. From this point of view transplantation of cells into the CNS may have advantages as the CNS is, to at least some extend, immune privileged.

1.2 Defining stem cells

For the purpose of this study, it is important to clearly distinguish between the terms neural stem cell, neural progenitor cell and neural precursor cell. The working definitions for this study therefore were:

- Neural stem cells (NSCs). A multipotent cell population that have the ability to self-renew over long time periods and are able to generate progenitors capable of producing neurons, astrocytes and oligodendrocytes. Self-renewal and multipotency are maintained for the entire life of the neural stem cell.
- Neural progenitor cells. A cell population with a limited self-renewal capacity that is fate restricted to give rise to either neurons, astrocytes or oligodendrocytes, respectively.
- Neural precursor cells (NPCs). These encompass both populations as a mixture of neural stem cell and neural progenitor cells.

These terms are often loosely used in the literature and the definitions used in this thesis are based on common considerations of what constitutes these cell populations (McKay, 1997; Morrison et al., 1997; Gage, 2000; Svendsen et al., 2001; Seaberg and van der, 2003).

1.3 Adult neurogenesis

"We are born with a certain number of brain cells which decrease with age. Everything must die in the brain or spinal cord – nothing can regenerate" (y Cajal, 1902). The existence of stem cells in the adult central nervous system has historically remained unnoticed, the brain being considered as a structure incapable of regeneration. The first evidence for neurogenesis in the adult CNS was published 40 years ago (Altman and Das, 1965; Lewis, 1968; Privat and Leblond, 1972), but the idea that new neurons are generated has found acceptance only in the last decade. This is mainly due to use of proliferating markers such as tritiated thymidine, 5-bromo-2'-deoxyuridine (BrdU), and retroviral labelling demonstrating *de novo* development of neurons in the adult mammalian brain (reviewed by Kuhn et al., 2001; Rakic, 2002; Picard-Riera et al., 2004). In mammals, including humans, proliferating precursor cells can be isolated from various regions of the adult brain, including human subcortical white matter (Nunes et al., 2003), cortex (Arsenijevic et al., 2001), hippocampus (Eriksson et al., 1998; Roy et al., 2000), subventricular zone (Kirschenbaum et al., 1994; Kukekov et al., 1999; Palmer et al., 2001) and cerebellum (Jain et al., 2003). However, new neurons are only added into restricted regions of the mammalian brain these being interneurons in the olfactory bulb and granule neurons in the hippocampus (for review see Doetsch, 2003). Other regions of the adult mammalian brain, such as the neocortex, have also been reported to generate new neurons (Gould et al., 1999; Magavi et al., 2000), but this has remained controversial (Kornack and Rakic, 2001; Koketsu et al., 2003). Functionality of new neurons has also been confirmed (van Praag et al., 2002; Carlen et al., 2002; Carleton et al., 2003). The new neurons derive from neural stem cells originating at two sites, the subventricular zone (SVZ) of the lateral ventricle wall and the dentate gyrus subgranular zone (SGZ) of the hippocampus.

1.3.1 Neurogenesis in the subventricular zone (SVZ)

The SVZ is the largest germinal zone of the adult mammalian CNS and comprises a layer of dividing cells extending along the lateral walls of the lateral ventricle (Figure 1-1). Throughout the SVZ new neurons are produced and feed cells into a stream of dividing cells forming the rostral migratory stream (RMS) that leads to the olfactory bulb where the cells mature and differentiate into granule and periglomerular cells (for references see Alvarez-Buylla and Garcia-Verdugo, 2002). While neurogenesis has been shown to occur in the olfactory bulb (OB) of mammalian species (Lois and Alvarez-Buylla, 1994; Bonfanti et al., 1997), including primates (Kornack and Rakic, 2001), its relevance still needs to be elucidated for the human OB considering that no signs of chain migration have so far been found (Sanai et al., 2004).

The SVZ from rodent species contains at least four distinct cell types based on morphology, ultrastructure, and molecular markers (Doetsch et al., 1997). A scaffold of astrocytes formed by slowly dividing type B cells allows young neuroblasts (type A cells) to migrate as homotypic chains (Lois and Alvarez-Buylla, 1994; Wichterle et al., 1997). Close to the chains of neuroblasts, clusters of rapidly dividing transit-amplifying type C cells are distributed that give rise to type A cells. The SVZ is separated from the lumen of the ventricle by a layer of multiciliated ependymal cells. Type B astrocytes occasionally contact the ventricle lumen by extending a short single cilium (Doetsch et al., 1999) between these ependymal cells that is similar to those found on embryonic neuroepithelial progenitor cells (Cohen and Meininger, 1987). Recently, the human SVZ was shown to contain a ribbon of astrocytes lining the lateral ventricle and separated by a gap from the ependymal cell layer (Sanai et al., 2004).

The origin and nature of the stem cell has remained a matter of controversy. Two cell types have been reported as the source for neural stem cell in the SVZ-ependymal region: a subpopulation of ependymal cells (Johansson et al., 1999), or type B astrocyte-like cells in the subventricular zone (SVZ) (Morshead et al., 1994; Garcia-Verdugo et al., 1998; Chiasson et al., 1999; Doetsch et al., 1999; Alvarez-Buylla et al., 2001). These insights have resulted from the ablation of faster dividing type A and

C cells using the antimitotic drug cytosine-b-D-arabinofuranoside (Ara-C), leaving exclusively type B cells proliferating (Doetsch et al., 1999). SVZ astrocytes were the only cells to divide after AraC treatment and started generating new type C cells, which in turn divided to give rise to type A cells. Labelling specifically SVZ astrocytes with an avian retrovirus further showed generation, migration and differentiation of labelled cells in the OB, as well as these cells showing self-renewal and multipotency *in vitro*. This has further been supported by studies showing that although ependymal and sub-ependymal cells can divide *in vitro*, only the subependyma-derived cells could self-renew and give rise to neurons and glia (Chiasson et al., 1999). This has lead to the suggestion that SVZ astrocytes are the *in vivo* primary precursor cells and act as stem cells *in vitro* (Doetsch, 2003).

Astrocytes outside the SVZ do not appear to be neurogenic *in vivo* under normal conditions, but astrocytes isolated from various brain regions before postnatal day 10 have chacteristics of neural stem cells *in vitro* (Laywell et al., 2000). However, the differences between them remain unclear. The precise mechanisms of controlled proliferation and restricted neurogenesis in the adult mammalian brain are still unknown, but it seems that the microenvironment in which these neural stem cells reside has an important impact on their behaviour. It is intriguing that classical developmental signals and morphogens, such as Notch, BMPs, Eph/ephrins, Noggin and sonic hedgehog have been suggested to play a role as maintaining and instructive factors in determining the particular "stem cell niche" (reviewed by Alvarez-Buylla and Lim, 2004; Fuchs et al., 2004).

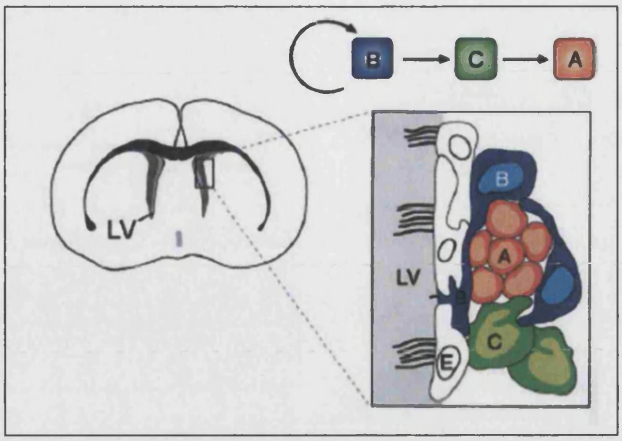


Figure 1-1: The SVZ as a germinal layer. Coronal section of a rodent brain indicating the location of the SVZ in the lateral wall of the lateral ventricle (LV). The magnification shows the cellular composition of the SVZ as type A cells (young neurons), type B cells (slowly dividing astrocytes), and type C cells (rapidly dividing transit-amplifying cell). (Adapted from Alvarez-Buylla and Garcia-Verdugo, 2002).

1.3.2 Neurogenesis in the subgranular zone (SGZ)

Neural precursor cells in the hippocampus were suggested to originate from the subgranular zone (SGZ) of the dentate gyrus (DG) and migrate only a short distance into the granule cell layer (GCL), where they differentiate into granule neurons that extend axons to the CA3 region (Altman and Das, 1965; Bayer et al., 1982; Kaplan and Bell, 1984; Kuhn et al., 1996). Although neural precursors were isolated from the adult hippocampus that exhibit neural stem cell characteristics *in vitro* (Gage et al., 1995; Palmer et al., 1995; Palmer et al., 1997), it has recently been shown that stem cell characteristics are limited to cells isolated from the hippocampus-adjacent regions of subependyma, but not of the adult DG that only contains neural progenitor cells (Seaberg and van der Kooy, 2002). Similar to the adult SVZ, astrocytes also play a role as neural precursor cells in the adult SGZ *in vivo* (Seri et al., 2001). Interestingly, astrocytes generated from the adult rat hippocampus are sufficient to instruct stem cells from newborn animals to convert into neurons (Song et al., 2002; Svendsen, 2002), an activity that seems to depend on the region the astrocytes were derived from. Future experiments might reveal the factors responsible for this.

1.3.3 The function of adult neurogenesis

The question arises of the function of the newly generated neurons in the olfactory bulb and hippocampus. An extensively studied, and indeed system *par excellence* for studying functional adult neurogenesis, is the song system of songbirds where neurons are continuously replaced and new neurons participate in plasticity and song learning (for review see Nottebohm, 2002). Nevertheless, hippocampal neurogenesis has also been observed in rodents (Kuhn et al., 1996), non-human primates such as macaques (Gould et al., 1999; Kornack and Rakic, 1999), marmosets (Gould et al., 1998), and humans (Eriksson et al., 1998). Potential roles in cognition and memory function are likely, and indeed it has been shown that neurogenesis increases in an enriched environment (Kempermann et al., 1997) and with exercise (van Praag et al., 1999) in the rodent brain. An interesting example suggesting that plasticity might have relevance for the human brain involved a study of London taxi drivers which showed increased hippocampal size in these subjects (Maguire et al., 2000). However, studying the function of hippocampal neurogenesis is not straightforward and although the hippocampus can be characterized as the "gateway to memory", it appears to be more involved in information processing rather than storage (Kempermann, 2002). Although the loss of hippocampal-dependent memories has been correlated with ablation of dividing cells and neurogenesis (Shors et al., 2001), the techniques employed leave the results open to interpretation (Macklis, 2001; Kempermann, 2002).

Much less is known about the function of new neurons in the OB of adult mammals. A continuous replacement of neurons in the OB may represent a refinement of existing olfactory circuitry with a changing environment, changing odours or a changing of their relevance. It has been shown that newly generated neurons in the OB responded to odorants as a physical stimulant by the upregulation of c-Fos expression demonstrating functionality of the cells (Carlen et al., 2002). Therefore, olfactory neurogenesis could be a form of adaptation to the environment, but might be species dependent considering the absence of the RMS in humans (Sanai et al., 2004), and that neurogenesis in the human olfactory bulb has not yet been shown.

Adult neurogenesis could also represent a mechanism of cell replacement or self-repair of the CNS. Such a prospective function could be stimulated by insults such as disease, as described for acute animal models in the following section.

1.3.4 Reactivation of precursors in the adult CNS

It has been demonstrated in several animal models that after inducing an acute or chronic insult of the CNS neural stem cells retain the capacity to be re-activated. Proliferation of neural precursor cells is increased after seizure (Parent et al., 2002), ischemia (Zhang et al., 2001; Nakatomi et al., 2002; Arvidsson et al., 2002), transection (Weinstein et al., 1996), nigrostriatal lesioning (Lie et al., 2002; Zhao et al., 2003) and also demyelination (Calza et al., 1998; Nait-Oumesmar et al., 1999; Picard-Riera et al., 2002). An increased cell proliferation has also been reported in the brains of patients affected with Huntington's disease (Curtis et al., 2003) and in non-human primates exposed to ischemia (Tonchev et al., 2003). Reactivation is often demonstrated by an increased number of BrdU labelled cells. However, BrdU labelling is not a unique marker for cell proliferation but rather incorporates into any cell with DNA synthesis including those undergoing DNA-repair and must therefore be carefully considered when it is to be used as marker for *de novo* developed cells (see Rakic, 2002). Some of the studies refered to confirmed maturation of the reactivated cells into post-mitotic differentiated neural cell types (Nait-Oumesmar et al., 1999; Arvidsson et al., 2002), but functional replacement has only rarely been demonstrated (Nakatomi et al., 2002). Although it is intriguing to suggest that the injured CNS has the potential to reactivate, and possibly even has a limited capacity for self-repair, these events must be rare considering the impact of CNS insults on human diseases. Nevertheless, one of the most promising therapeutic targets for neural stem cells has been the treatment of neurodegenerative diseases such as Parkinson's disease.

1.4 Fundamentals of Parkinson's disease and its therapy

Parkinson's disease is, after Alzheimer's disease, the second most common age-related neurodegenerative disease. It is a progressive disease with a mean onset age of 55. The rate of incidence markedly increases with age (reviewed by Dauer and Przedborski, 2003) and as such affects about 1% of the population over the age of 65 (Shastry, 2001). The disease was first described by James Parkinson (1817) in his classic paper "An essay on the shaking Palsy" where he describes his observations of six patients. It took another century before any further relevant information was added to this work.

1.4.1 Clinical characteristics of Parkinson's disease

SHAKING PALSY (Paralysis agitans) Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward and to pass from a walking to a running pace: the senses and intellects are being uninjured (Parkinson, 1817).

The major symptoms of PD – tremor, bradkinesia (slowness of movement), muscle rigidity, postural defects and freezing (the inability to begin a voluntary movement), significantly decrease the quality of life of patients. Often these cardinal symptoms are accompanied by the inability to communicate with the environment due to paucity of normal facial expression (hypomimia), decreased voice volume (hypophonia), drooling (failure to swallow without thinking about it), decreased size and speed of handwriting as well as abnormalities of affect and cognition (reviewed by Dauer and Przedborski, 2003). Depressions are common, and often, particularly older patients, develop dementia. Over time, symptoms worsen and prior to availability of treatment the mortality rate among PD patients was three times higher compared to normal age-mached subjects.

1.4.2 Neuropathology of PD

The main neuropathological features of PD are the formation of fibrillar cytoplasmic inclusions in the substantia nigra, so called Lewy bodies (Lewy, 1912; Tretiakoff, 1912), and the profound depletion of dopaminergic neurons in the nigrostriatal system (Bernheimer et al., 1973; McGeer et al., 1988).

A crucial role of α-synuclein in the pathogenesis of PD has not just come from the discovery of mutations in the gene in some familial syndromes (see below), but that α-synuclein is also a prominent constitutent of Lewy bodies in idiopathic PD (Goedert and Spillantini, 1998; Mezey et al., 1998). α -synuclein is widely expressed in the nervous system and its normal physiological role is just beginning to be elucidated, but it appears to play a role in modulation of synaptic vesicle function (reviewed by Kahle et al., 2002). Although it is normally a soluble unfolded protein, it can aggregate into insoluble amyloid fibrils which then form Lewy bodies, followed by subsequent ubiquitination and accumulation of neurofilaments (Goedert and Spillantini, 1998; Mezey et al., 1998). The presence of Lewy bodies is not exclusive to PD, and is also relevant to other neurodegenerative diseases. However, the presence of Lewy bodies in the substantia nigra in conjunction with the nigral cell loss is the common hallmark of sporadic PD (Dunnett and Bjorklund, 1999) and suggests a neurotoxic role contributing to neurodegeneration in PD. Overexpression of wild-type α-synuclein by a genomic triplication causes the disease in a pedrigree of familial PD (Singleton et al., 2003). A number of cellular and transgenic animal models of wild-type α-synuclein-induced neurodegeneration have been developed (reviewed by Dawson et al., 2002) and have started to shed light on the pathogenic mechanisms that underly the formation of prospective neurotoxic fibrils in idiopathic PD. It has been suggested that inhibition of mitochondrial complex I creates an environment of oxidative stress that ultimately leads to aggregation of α-synuclein. It is thought that aggregated α-synuclein would then bind to proteasomes, inhibit ubiquitin-dependent proteasomal function preventing clearance of proteins that were destined for degradation, and ultimatively induce dopamine neuron cell death (Dawson and Dawson, 2003).

At the onset of symptoms, around 70-80% of the striatal dopamine (Bernheimer et al., 1973) and about 50% of nigral dopamine neurons (Fearnley and Lees, 1991) have already been lost. Therefore, it appears that a threshold of dopamine neuron loss has to be passed before symptoms become apparent. Different models have been proposed to explain the progressive cell death in PD (see Dunnett and Bjorklund, 1999). In the accelerated "ageing model", an elevated rate of cell death occurs where cell loss starts early and progresses more rapidly so that the critical level of dopamine loss is reached during the normal life span. In the "assault model" an acute insult causes an immediate but partial loss of dopaminergic neurons, followed by the normal age-dependent rate of degeneration. Nigral cell loss is not exclusive to the diseased brain but is a process that also happens during normal ageing at a rate of about 5% per decade (Fearnley and Lees, 1991). However, in PD patients there is from the onset of symptoms an exponential progress of cell death at a tenfold higher rate (45% per decade) (Fearnley and Lees, 1991). Hence, cell loss is likely to start 4-5 years before symptoms appear and considering that most cases of sporadic PD begin at middle age, an exponential degeneration has been favoured (Dunnett and Bjorklund, 1999). It has also been hypothesised that individuals born with a smaller number of nigral neurons, e.g. due to exposure to environmental factors, might be more susceptible to reaching the critical level of neuronal loss during the normal lifespan (Di Monte, 2003; Barzilai and Melamed, 2003).

Neuropathalogical studies have shown that PD-associated loss of dopaminergic neurons is distinct from the pattern seen in normal ageing and cell loss is concentrated in ventrolateral and caudal portions of the substantia nigra pars compacta (SNpc), whereas during normal ageing the dorsomedial aspect of SNpc is affected (Fearnley and Lees, 1991). It has also been suggested that striatal dopaminergic nerve terminals are the primary target of the degenerative process (Dauer and Przedborski, 2003) which is accompanied by the accumulation of aggregated proteins in nigral processes known as Lewy neurites (Lotharius and Brundin, 2002). In this hypothesis dopamine neurons are "dying backwards" in a retrograde degeneration, followed by further accumulation of aggregated proteins in nigral cell bodies (Lewy bodies), and, finally culminating in cell death (Lotharius and Brundin, 2002; Dauer and Przedborski, 2003). Interestingly, dopamine itself might play a role in neurodegeneration in this

model. Toxic forms of α-synuclein can permeabilze vesicles and thereby cause leakage of small molecules, such as dopamine, out into the cytoplasm. Subsequently this could lead to oxidative stress primarily at nigrostriatal terminals, where most of the neurotransmitter is produced and stored (Lotharius and Brundin, 2002).

1.4.3 Etiology of Parkinson's disease

The causes of PD are unknown, although it is believed that a combination of genetic (Muenter et al., 1998; Gasser, 2001; Valente et al., 2001) and environmental factors (Langston and Ballard, Jr., 1983; Betarbet et al., 2000) may be responsible for the majority of PD cases. Therefore, a combination of genetic predisposition and exposure to environmental factors may implicate differences in the individual susceptibility to metabolise dopamine-related neurotoxins. In about 95% of PD cases no apparent genetic linkage exists and the disease is referred to as "sporadic" PD (Dauer and Przedborski, 2003).

1.4.3.1 Environmental hypothesis for PD

The environmental hypothesis postulates that PD-related neurodegeneration is a result from exposure to dopaminergic neurotoxins. A compelling prototypic example that exogenous toxins can induce PD was demonstrated by the discovery that the protoxin *n*-methyl-4-ohenyl-1,2,3,6-tetrahydropyridine (MPTP) causes PD-like syndromes in humans (Langston and Ballard, Jr., 1983). Other environmental toxins have been shown to induce a parkinsonian state, such as the herbicide paraquat and the insecticide rotenone (reviewed by Di Monte, 2003). All of the toxins have in common that they induce oxidative stress and/or inhibit mitochondrial function and have been used to induce parkinsonism in rodent and primate to model the human disease (Beal, 2001).

Since MPTP is highly lipophilic it can easily cross the blood-brain barrier (Markey et al., 1984) and is then converted by monoamine oxidase B into its active form 1-methyl-4-phenylpyridinium (MPP^+) (Di Monte et al., 1996). Glial cells and serotonergic neurons play a role in this activation step as they catalyse this oxidation.

A selective function on degeneration of dopaminergic neurons might be due to its high affinity for the plasma-membrane dopamine transporter (Javitch et al., 1985) by which it is taken up into the cell soma. MPP^+ also has affinity for the vesicular monoamine transporter (VMAT2) (Liu et al., 1992). This decreases vulnerability to the toxin by sequestering MPP^+ and sequential storage into synaptic vesicles. Alternatively, MPP+ can be taken up and concentrated in mitochondria where it is an inhibitor of mitochondrial complex I (Nicklas et al., 1985; Ramsay and Singer, 1986) which might increase free radical production (Chan et al., 1991). Although MPTP toxicity in primates and humans mimics the clinical signs of PD, including tremor, rigidity, akinesia and postural defects (Bloem et al., 1990), it does not induce the formation of Lewy bodies (Forno et al., 1986) and its action is far more acute, causing PD within a few days after MPTP administration, rather than progressive. However, MPP^+ toxicity has shown that mitochondrial dysfunction causes PD-like symptoms, which is supported by observations of complex I deficiency in the substantia nigra of PD patients (Schapira et al., 1990), an effect which is specific to PD, and not seen in any other diseases affecting the same neurons (Dunnett and Bjorklund, 1999). The primate MPTP model is the gold standard for the assessment of novel strategies and agents for the treatment of PD symptoms (Dauer and Przedborski, 2003).

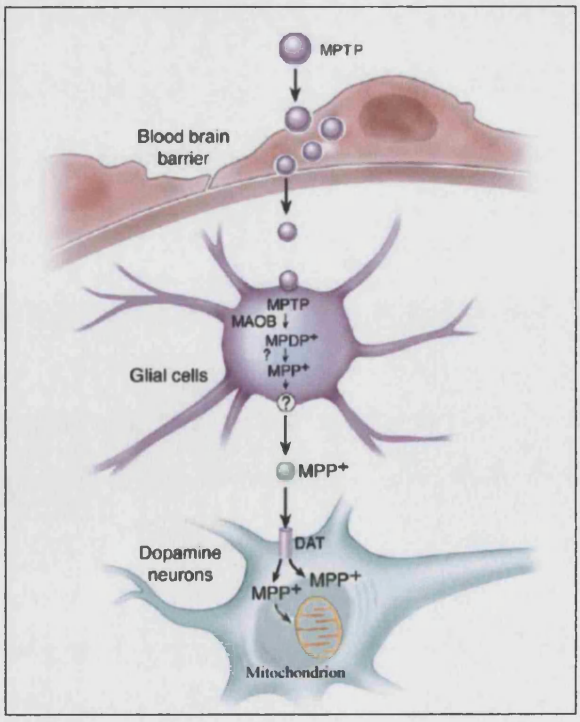


Figure 1-2: Neurotoxicity of MPTP metabolism. (Adapted from Dauer and Przedborski, 2003).

Similar to MPP^+ is the effect of rotenone on inhibition of mitochondrial complex I. Continuous intravenous injections of rotenone into rats causes a progressive and selective degeneration of dopaminergic neurons (Betarbet et al., 2000) with PD-like symptoms such as bradykinesia, postural instability, disturbance of gait, some evidence of tremor and most interestingly cytoplasmic inclusions that stain with antibodies for ubiquitin and α-synuclein. However, the originally proposed selective toxicity of rotenone for dopaminergic neurons is in contrast to recent studies demonstrating significant reductions in striatal DARP-32-positive neurons, cholinergic, and NADPH diaphorase-positive neurons (Hoglinger et al., 2003). It has been suggested that dopaminergic neurons might be more susceptible to toxin-induced degeneration and particularly vulnerable to complex I inhibitors (Beal, 2001; Di Monte, 2003).

On the other hand environmental factors can also exhibit protective effects and in epidemiological studies, for example, it has been shown that caffeine consumption (Ross et al., 2000; Ascherio et al., 2001) and cigarette smoking (Morens et al., 1995; Sugita et al., 2001) can decrease the risk of PD.

Disruption of the normal cell metabolism might also contribute to neurodegeneration as an inadequate degradation of metabolic products could result in an accumulation of neurotoxic products. One source of endogenous toxins might even result from dopamine metabolism itself, in which harmful reactive oxygen species (ROS) are generated (Cohen, 1984). With respect to studies investigating the role of α-synuclein in neurodegeneration, evidence has implicated a role of α-synuclein, dopamine, and ROS in selective degeneration mechanism of nigral dopamine neurons in PD (Steece-Collier et al., 2002).

The study of environmental risk factors causing PD is difficult, since exposure to toxins may have occured long before onset of the disease. In addition, symptoms of PD may be the result of additive or synergistic effects of multiple exposures to environmental toxins. Although human epidemiological studies have implicated residence in a rural environment and related exposure to herbicides and pesticides

with an elevated risk of PD (Tanner, 1992), there is as yet no convincing data to implicate any specific toxin causing PD (Dauer and Przedborski, 2003). However, toxins have been hypothesised to predispose to the development of PD rather than causing evident symptoms (Di Monte, 2003). Toxins may still cause a discrete loss of dopaminergic neurons and symptoms then becoming obvious after subsequent or additive exposure resulting in a synergistic damage of the nigrostriatal system (Thiruchelvam et al., 2000).

1.4.3.2 Genetic hypothesis for PD

A genetic component in PD has not generally thought likely since epidemiological studies examining twins suggested an absence of genetic factors (Tanner et al., 1999; Piccini et al., 1999), and also due to the fact that most patients had a sporadic disease. However, twin studies have been the cause of much debate as parkinsonian signs are not necessarily apparent in persons with partial nigrostriatal degeneration. Therefore, presymptomatic PD can only be assessed by positron-emission tomography with fluorodopa [^{18}F]. This revealed a significantly increased rate of concordance when abnormal striatal dopaminergic uptake was measured in the otherwise asymptomatic twin of a discordant pair (Morrish et al., 1998). Further evidence for potential genetic factors has come from the discovery of inherited cases of PD (Payami et al., 1994; Marder et al., 1996; Piccini et al., 1997) and by the observation that monozygotic twins with an onset of disease before the age of 50 years do have a very high rate of concordance (Tanner et al., 1999). However, the real advance has occurred with the discovery that the rare monogenetically inherited forms of the disease are linked to mutations in the α-synuclein gene (Polymeropoulos et al., 1997; Kruger et al., 1998), parkin gene (Ishikawa and Tsuji, 1996), the gene for ubiquitin hydrolyase L1 (Leroy et al., 1998) and the gene for DJ-1 (Bonifati et al., 2002). Interestingly, all of the PD genes identified have in common that they appear to participate in the ubiquitin-proteasome pathway which is particularly compelling with respect to Lewy body aggregates that are pathologically defining for sporadic forms of the disease.

1.4.3.3 Neurodegeneration as a lack of adult neurogenesis?

A hypothesis has been postulated that a primary deficit in neural stem cell proliferation, migration and differentiation might contribute to net cell loss and neuronal circuit disruption in neurodegenerative diseases (Armstrong and Barker, 2001). Here, the failure of endogenous neural stem cells to respond normally and appropriately to cell loss in neurodegenerative diseases, and thereby to replace neurons that die, would contribute to progression and thus pathogenesis of e.g. Parkinson's disease. Recent findings suggested neurogenesis in the adult rodent substantia nigra (Zhao et al., 2003), where the absolute number of dopaminergic neurons remained constant throughout the lifetime of the animals thus implying that new neurons are delivered to this region to replace those that are lost. Is neurodegeneration simply a disruption of this normal homeostasis in some predisposed patients? Would disruption of neural stem cell genesis otherwise cause neurodegeneration? It is noteworthy to mention anecdotal reports of several patients developing parkinsonism with radiographic evidence of basal ganglia atrophy after whole body irradiation and high dose antimitotic chemotherapy (Mott et al., 1995). Considering recent findings demonstrating the lack of neuronal turnover and/or replacement of injured neurons in the human brain (Sanai et al., 2004), it has shown that species differences are an important issue to be considered in finding the right model to test this hypothesis. However, the identification of the cellular and molecular mechanisms that prevent neural stem cells from becoming integrated into functional neuronal networks could indeed be a major achievement (Rakic, 2004).

1.4.4 Therapeutic approaches for Parkinson's disease

Since the cause of PD still needs to be elucidated and most cases of the disease are sporadic, therapies for PD are symptomatic. However, with recent progress in neuroimaging a decline in striatal dopamine function can be detected before clinical symptoms appear (Sanchez-Pernaute et al., 2002), which may open the way to novel neuroprotective therapies to block or slow down the degenerative process.

The major breakthrough in the treatment of PD was the discovery that administration of the dopamine precursor L-DOPA alleviates symptoms of akinesia (Carlsson et al., 1957; Birkmayer and Hornykiewicz, 1961) and improves the daily life of patients in the early stages of the disease significantly. However, these benefits in the early phases were set back by a decline in the efficacy of the drug ("wearing-off effect") as the disease progresses, and severe site effects occur, most importantly dyskinesia, within 5 years with beginning of the treatment (Duvoisin and Marsden, 1974; Rascol et al., 2000; Bezard et al., 2001). Although, the main treatment of PD is still the oral administration of levopoda (Djaldetti and Melamed, 2001), the problems accompanied with the use of this drug have led to the development of neurosurgical approaches (Follett, 2000), neuroprotective therapies (Dunnett and Bjorklund, 1999), cell replacement therapies (Bjorklund and Lindvall, 2000), and gene therapeutic approaches (Bohn, 2000).

1.4.4.1 Neurosurgical therapies

Surgical treatments for PD include ablative techniques (thalamotomy and pallidotomy), augmentative techniques (deep brain stimulation), and restorative techniques (tissue transplantation and gene therapy) (Follett, 2000). Restorative techniques will be considered separately.

Surgical treatment for the relief of symptoms in PD patients was historically used as early as 1912 (Guridi and Lozano, 1997; Speelman and Bosch, 1998) and intensified as pallidal and thalamic lesioning in the 1940s and 1950s (Laitinen et al., 1992) but lost interest with the introduction of L-DOPA in the 1960s. With advances in neurosurgical procedures accompanied by lower morbidity rates and with the awareness of site effects involved with L-DOPA treatment, surgical procedures have now experienced a renaissance. However, functional neurosurgery is purely symptomatic and only given in patients with severe motor fluctuations or L-DOPA-induced dyskinesias where no further improvement can be achieved with adjustments in medications (Betchen and Kaplitt, 2003).

Based on the anatomy and physiology of the basal ganglia and motor systems, neurosurgical interference targets the ventral intermediate nucleus of the thalamus, the globus pallidus (GPi) and the subthalamic nucleus (STN). Due to the loss of inhibition from the putamen, an increased activity of neurons in the STN causes an overactivity of neurons in the GPi and substantia nigra reticular in PD patients. Overactivity from these regions subsequently exert inhibitory influences on thalamic and brainstem nuclei that are responsible for hypokinetic symptoms of PD (rigidity and bradykinesia). Therefore, pallidal interventions are aiming to reduce GPi activity and to restore normal thalamocortical and brainstem activity. Pallidotomy has significantly improved L-DOPA-induced on-dyskinesias with improvements in the UPDRS dyskinesia scale (Follett, 2000) but are usually performed unilaterally due to the risk of complications. Alternatively to pallidal procedures non-destructive techniques, such as deep brain stimulation (DBS), have been developed that produce adjustable and reversible functional lesion. The STN has become a popular target for DBS in patients who have severe motor fluctuations, dose-limiting dyskinesias or tremors (see Betchen and Kaplitt, 2003).

1.4.4.2 Neuroprotective therapies

The progressive cell death in PD involves a cascade of events, such as oxidative stress, mitochondrial dysfunction, excitotoxicity, and inflammatory changes, that all lead to apoptotic and necrotic cell death (for reviews see Olanow and Tatton, 1999; Dunnett and Bjorklund, 1999). Neuroprotective therapies aim to delay this cell death in order to maintain functional neurons as long as possible.

Clinical trials have been performed using antioxidants, such as selegiline hydrochloride (Shoulson, 1998) and dopamine receptor agonists (for review see Shults, 2003), the latter stimulates dopamine autoreceptors and thereby reduces dopamine synthesis, turnover and release, so that less L-DOPA is needed.

A hallmark for neuroprotective therapies was the discovery of neurotrophic factors, and their survival and rescue effects on injured neurons after damage in the adult brain. A promising candidate in neuroprotection and regeneration is glial cell-line

derived neurotrophic factor GDNF (Engele and Bohn, 1991), which facilitates strong survival effects on dopaminergic neurons *in vitro* (Lin et al., 1993) and *in vivo* after mechanical (Beck et al., 1995) or toxic (Hoffer et al., 1994) insult. Several studies have shown behavioural improvement after intrastriatal, intranigral or intracerebroventricular injections of recombinant GDNF protein in rodent (Rosenblad et al., 1999; Kirik et al., 2000) and primate (Gash et al., 1996; Kordower et al., 2000) models of PD. GDNF has three different effects on dopaminergic neurons: (i) it rescues damaged neurons when given before or shortly after the insult; (ii) it promotes axonal sprouting or regeneration in lesioned animals; (iii) it stimulates dopamine turnover and function in dopaminergic neurons (Dunnett and Bjorklund, 1999; Kirik et al., 2004). In the first double-blind placebo controlled clinical study, PD patients received monthly intracerebroventricular injections of recombinant GDNF (Kordower et al., 1999). However, the study failed to improve clinical symptoms or to prevent nigrostriatal degeneration due to an ineffective delivery system. The poor penetration of the blood-brain barrier, as well as limited passage of recombinant proteins from the cerebrospinal fluid into the brain tissue, requires other routes of administration directly to the receptive target sites. Animal models have already demonstrated that beneficial effects of GDNF strongly depend on where it is administrated (Kirik et al., 2004). In rodent and primate models it has been demonstrated that GDNF is not only neuroprotective to residual dopamine neurons but can also restore neurochemical function by increased dopamine turnover and/or release and also induces aberrant most likely functional sprouting (Kirik et al., 2004). Recently, new studies administrating recombinant GDNF protein continuously in the caudate putamen of PD patients have promising results (Gill et al., 2003). The data indicated significant improvement in off-medication motor sub-score of the UPDRS and daily living sub-score without any serious side effects. The early onset of symptomatic improvement and the small increase in [^{18}F]-DOPA uptake around the injection site indicate a functional upregulation in residual dopaminergic neurons, rather than a regenerative response. Although this open-label trial was based only on a small number of subjects (5 patients) and monitored over a relatively short follow-up period (1 year) it is very promising for future clinical trials and those that are underway (Svendsen, personal communication).

Continuous delivery of recombinant GDNF has potential problems, including complications associated with the chronically implanted infusion pump (Gill et al., 2003) and alternative routes of delivery have been considered. Other approaches for the efficient delivery of GDNF are using encapsulated genetically engineered cells (Tseng et al., 1997), viral vectors (for review see Kirik et al., 2004) or engineered neural stem cells (Akerud et al., 2001). In a non-human primate model of the disease, lentiviral delivered GDNF showed significant improvement in functional recovery and a complete prevention of nigrostriatal degeneration (Kordower et al., 2000). Neurotrophic effects on dopaminergic neurons were also shown for other factors belonging to the GDNF family, such as neurturin (Horger et al., 1998), artemin (Masure et al., 1999), and persephin (Milbrandt et al., 1998).

1.4.4.3 (Stem) Cell based replacement strategies for Parkinson's disease

Cell replacement strategies for Parkinson's disease are based on the idea that neural grafts restore dopamine neurotransmission in the striatum and lead to substantial and long-lasting functional recovery. Therefore, the success of cell replacement therapies for Parkinson's disease relies on two hypotheses: (1) the predominant symptoms of PD are dependent on the dysfunction or loss of dopaminergic neurons in the nigrostriatal pathway; and (2) dopaminergic neurons grafted into the dopamine-deficient striatum can replace those neurons lost as a result of the disease and can reverse some of the major symptoms of the disease (Bjorklund et al., 2003). Considering the complexity of brain circuitry the functional and structural integration of the graft into the depleted system appears a remote possibility. Functional integration of neuronal cells involving the establishment of reciprocal connectivity and formation of axonal connections are mainly restricted to the developing brain. However, functional integration of grafted neurons have been demonstrated in the adult brain of rodents and the capacity is increased after damage suggesting that mechanisms regulating neuronal differentiation and connectivity during development may be reactivated by lesions or degenerative changes (reviewed by Bjorklund and Lindvall, 2000). However, it can only be speculated if this is also relevant for the human adult brain. On the other hand, reconstruction of neural circuitry is not necessarily a prerequisite for functional recovery: transplated cells may release the

missing neurotransmitter dopamine in a tonic manner, or they may produce neurotrophic or neuroprotective factors that can counteract degeneration or promote regeneration.

The basic proof of principle for cell replacement therapies has been provided by extensive animal experiments. Although these rodent and primate models of PD have a different etiology from the human disease (the lesion is acute rather than progressive), they still mimic its cardinal features, the depletion of dopaminergic neurons in the substantia nigra. Such experiments have shown that immature dopamine neuroblasts survive and re-establish dopaminergic innervation when transplanted into the denervated striatum, and restore baseline dopamine synthesis and release in the reinnervated area (reviewed by Bjorklund and Lindvall, 2000). Most of these studies have used ectopic locations of the graft (transplanted cells were placed into the striatum rather than into the substantia nigra) and are therefore unlikely to receive normal afferent inputs. This ectopic placement is necessary for extensive reinnervation of the striatum by the transplanted cells and they function by tonic release of dopamine at both synaptic and non-synaptic sites.

The successful functional recovery by foetal transplants in animal models of PD was justification for a series of clinical trial in humans since 1987. The first phase of clinical trial comprised small open-labelled trials that gave evidence that foetal transplants can survive and function for more than 10 years in PD patients and revealed therapeutic benefits, such as an increased time in the "on" phase (that is the time with a few or no motor symptoms), improvements in speed and accuracy of movement in defined "off"-phase (after drug withdrawal) and significant increase in striatal [^{18}F]-DOPA uptake (reviewed by Dunnett et al., 2001). However, the main issues with these open-labelled studies is a high variability of the results and clinical improvements may be due to investigator bias or placebo effects that are well known to occur in PD and can be long lasting (at least up to six months) (Kordower et al., 1995).

Based on the encouraging results from these open-labelled trials, the National Institute of Health (NIH) supported two double-blinded sham-surgery controlled trials in the

early 1990s. The outcome has been published and revealed rather disappointing results (Freed et al., 2001; Olanow et al., 2003). Two major concerns have been raised: only modest benefits in patients under the age of 60 (no improvement in patients over the age of 60 years) and a significant number of patients developed severe off-state dyskinesias. In the study of Freed *et al.* (2001) patients received mesencephalic tissue from aborted human embryos (age 7-8 weeks after conception) that was prepared as 200μm large strands and was stored for up to four weeks before use. Four needle passes were made through the forehead and projected through the long axis of the putamen. Patients did not receive immunosuppression. One year after transplant [^{18}F]-DOPA PET scans showed survival of grafts in 85% of patients. Two post-mortem cases revealed that a rather low amount of 7,000-40,000 tyrosine hydroxylase-positive cells had survived. Only some of the younger patients showed improvements in UPDRS motor scores, but no change in older patients or in the sham-surgery group was observed. 15% of the patients developed severe dyskinesias, which persisted despite substantial reductions in levopoda doses. Similar results were obtained by the study from Olanow *et al.* (2002) showing no significant overall improvement in UPDRS motor scores, dyskinesias in more than half of the patients but some improvement in patients with less severe disease. Major criticism has been expressed about the conceptualization and conduct of these double-blinded studies, such as patient selection, preparation, storage and location of graft, lack of immunosuppression, surgical procedure and clinical assessment (Dunnett et al., 2001; Bjorklund et al., 2003). In the light of these studies the value of foetal transplants needs to be reassessed, methods improved and therapies tailored more individually to patients. A main technical problem and ethical concern is the availability of primary dopaminergic neurons. Considering the large amount of foetal tissue required for transplantation, this method will never be available as therapy for all patients with advanced PD and therefore, cannot meet its social goal.

For this reason neural stem cells and neural precursor cells, which potentially provide an unlimited supply of cells for regenerative therapies, have attracted great interest. This potential is based on their defining characteristics of self-renewal and multipotency. As stem cells by definition can be expanded over unlimited periods and retain the capacity to differentiate into different neural cell types, the challenge is to

guide their maturation into dopamine releasing cells or dopaminergic cell types that would be available for transplantation as required. This has the additional advantage that such cells can be standardized, screened and manipulated in ways that could never be possible with foetal tissue. Stem cells can principally be predifferentiated *in vitro* to dopaminergic neurons prior to transplantation or alternatively transplanted as undifferentiated neuroblasts that differentiate *in vivo* to dopaminergic neurons. The latter approach may have the advantage that the cells integrate better as compared to differentiated dopaminergic neurons and, in the ideal scenario, reconstruct the nigrostriatal pathway. However, this would require that the cues to instruct the immature precursor cell to differentiate into the missing dopaminergic neuron are available in the PD patient's brain. Transplantation of naive precursor cells to the adult striatum leads to predominantly glial differentiation (reviewed by Perrier and Studer, 2003), while "priming" prior to transplantation might allow precursor cells to adopt region-specific differentiation in the forebrain (Wu et al., 2002).

Potential cell sources for the generation of dopaminergic neurons are embryonic stem cells, neural stem cells from the embryonic or adult brain, or stem cells derived from other non-neural cells (Figure 1-3). Although the major goal is to generate a large number of functional dopaminergic neurons from these different sources, it is still an unresolved issue whether non-dopaminergic neurons and glial cells, that are normally present in the grafts used for transplantation so far, are important for the differentiation, survival and function of the grafted dopaminergic neurons. Therefore, a pure population of predifferentiated dopaminergic neurons may not necessarily give the optimal therapeutic benefit.

Recent excitement in the field suggested generation of neuronal cell types from other tissue sources by transdifferentiation (Woodbury et al., 2000; Brazelton et al., 2000; Mezey et al., 2000; Kohyama et al., 2001). Reports have suggested generation of dopaminergic neurons from dermis (Toma et al., 2001), bone marrow stromal cells (Li et al., 2001) and mesenchymal cells (Jiang et al., 2002). However, significant doubts about the neuronal identity and function of transdifferentiated cells have been raised (Anderson et al., 2001). Such easily accessible stem cell populations would

provide an ideal source for transplantation therapy, as the cells could be harvested from the patients own tissue and be used to generate neurons for transplantation.

With the discovery that neural stem cells exist in the mammalian brain and with the knowledge that we have acquired over the past two decades about their isolation and propagation, a hypothetical approach is to remove the cells from the patient, to expand and predifferentiate them *in vitro*, followed by reimplantation to replace the lost dopaminergic neurons. Thereby the risk of an immune reaction would be avoided. However, several problems require to be overcome: (i) it has not been shown that adult human stem cells can be expanded and converted in sufficient numbers; (ii) it is not known if cells from patients with neurodegenerative disease function similar to cells from an unimpaired brain. Similar problems have also to be considered in alternative approaches to stimulate endogenous stem cells. Injury of the CNS has been shown to recruit endogenous stem cells in a model of stroke (Nakatomi et al., 2002; Arvidsson et al., 2002), and recently evidence for neurogenesis in the adult substantia nigra has been suggested (Zhao et al., 2003) that is further increased after lesion. These findings will be discussed in more detail in later chapters.

As an alternative source to human foetal dopaminergic neurons, xenografting has been considered involving transplantation of tissue from one species into another. Although the use of porcine cells has advantages, such as big litters and the potential for greater cell migration and axonal outgrowth in the adult CNS (Hurelbrink et al., 2002), there exist disadvantages limiting its therapeutic application. Such major disadvantages are the risk of zoonotic infection involving the transmission of porcine endogenous retroviruses, rejection of xenografts by cellular and humoral processes, and limited data on functional capacity and capabilities of xenografts (reviewed by Bjorklund et al., 2003). Despite these concerns clinical trials using porcine xenografts for the treatment of PD have been undertaken (Schumacher et al., 2000).

Immunological problems accompanying transplantation of allogenic and xenogenic tissues and cells are in theory less of a concern for cells derived from somatic cell suclear transfer (SCNT). The concept, known as "therapeutic cloning", refers to the transfer of the nucleus of a somatic cell into an enucleated donor oocyte (Lanza et al.,

1999) and has been shown for different species (Cibelli et al., 1998; Munsie et al., 2000; Wakayama et al., 2001), including most recently the derivation of human ES cells (Hwang et al., 2004) from SCNT. Functional dopaminergic neurons were obtained from mouse ES cells via SCNT from adult tail tip or cumulus cells (Wakayama et al., 2001) and have recently been shown to improve impairment after transplantation into hemiparkinsonian mice (Barberi et al., 2003).

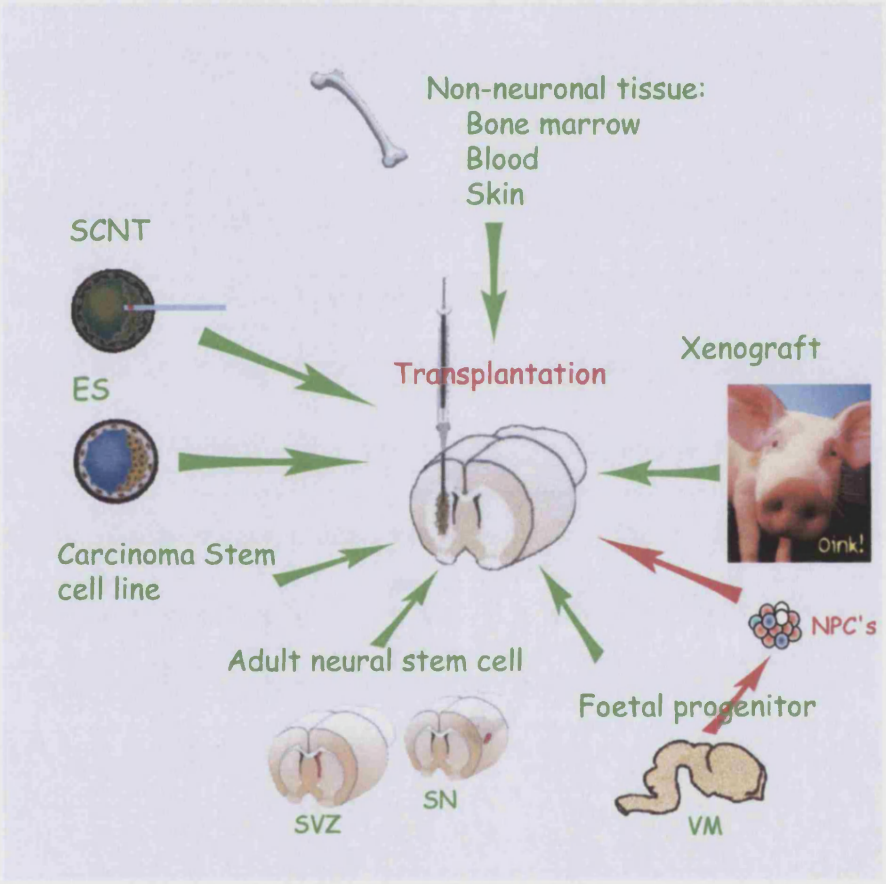


Figure 1-3: Cell sources for the generation of dopaminergic neurons. (Adapted from Lindvall, 2003).

1.5 Generation of dopaminergic neurons from neural precursor cells *in vivo* and *in vitro*

The following section will describe the signals known to be involved in the embryonic development of dopaminergic neurons and their relevance as instructive cues for the generation of dopaminergic neurons from neural precursor cells and embryonic stem cells.

1.5.1 Characterization of the dopaminergic system

Dopaminergic neurons belong to the group of catecholaminergic neurons that are characterized by the synthesis of the neurotransmitter dopamine. Dopamine is synthesised in two enzymatic steps from the amino acid tyrosine (Figure 1-4). Tyrosine hydroxylase (TH) catalyses the rate-limiting step in dopamine biosynthesis, the conversion of tyrosine to L-DOPA. Subsequently L-DOPA is decarboxylated to dopamine by aromatic L-amino acid decarboxylase (AADC). Dopamine is also used as a precursor for other catecholaminergic neurons, i.e. noradrenergic and adrenergic neurons, that all share the first two steps of the catecholamine-synthesis pathway leading to dopamine. Functional dopamine neurotransmission further depends on transport, synaptic packaging, re-uptake and degradation of dopamine (Figure 1-4) (Goridis and Rohrer, 2002; Lin and Rosenthal, 2003; Perrier and Studer, 2003). Characterization of neurons solely by the expression of TH does not therefore necessarily identify dopaminergic neurons, and other markers that are unique for midbrain dopaminergic neurons, such as dopamine transporter (DAT), aldehyde dehydrogenase-2, Nurr1, Lmx1b, Ptx3 and engrailed-1 (see all below), as well as morphological criteria and exclusion of the expression of dopamine β-hydroxylase characterizing adrenergic and noradrenergic neurons, are required (Arenas, 2002).

Dopaminergic neurons are organised in distinct groups and are found in the olfactory bulb, the retina, the diencephalon, but most prominently in the ventral midbrain where they are located in the substantia nigra (SN), the ventral tegmental area (VTA) and the retrorubral field (Nelson et al., 1996). They define topographically well-defined

projections: in rodents dopaminergic neurons from the SN project via the nigrostriatal system to the striatum (caudate-putamen in primates) and degeneration of this pathway is directly involved with motor symptoms in Parkinson's disease patients, while neurons from the VTA connect via the mesolimbic pathway to the limbic system and cortex and are implicated in obsessive-compulsive disorders, schizophrenia and substance abuse (Egan et al., 1996; Swanson et al., 1998).

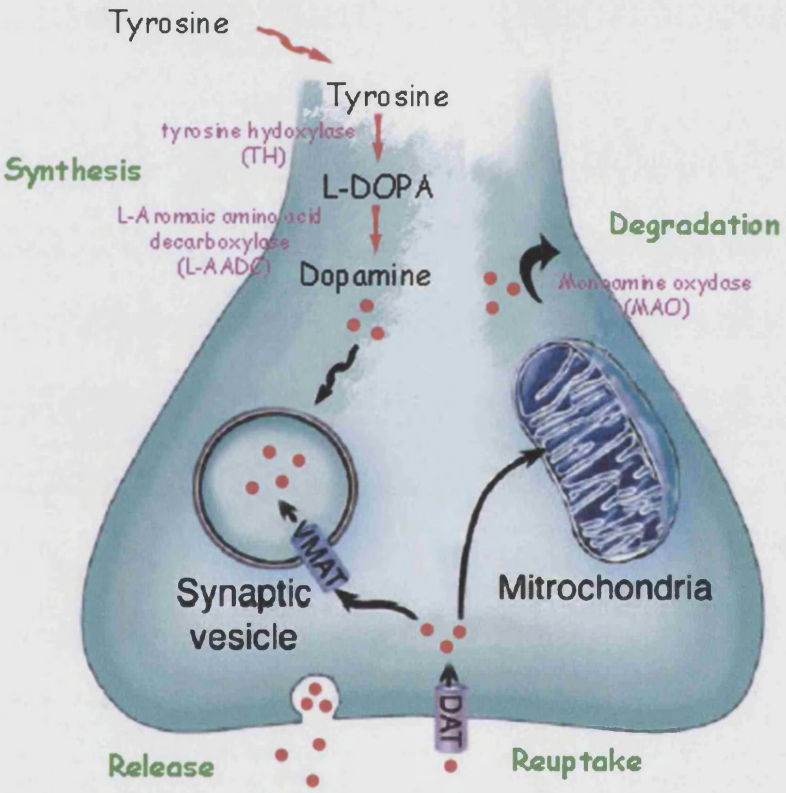


Figure 1-4: Simplified picture of dopamine metabolism. Plasma membrane dopamine transporter (DAT), vesicular monoamine transporter (VMAT). DAT terminates action of dopamine by rapidly removing it from the synapse. VMAT loads cytoplasmatic dopamine into vesicles for storage and subsequent release. (Adapted from Cooper et al., 1996).

1.5.2 Dopamine neuron development *in vivo*

Dopaminergic neurons are generated in the ventral mesencephalon as a result of a well-coordinated expression of morphogens, transcription factors and survival factors involving a cascade of steps (Figure 1-5):

1. Induction of an immature dopamine phenotype
2. Specification into differentiated dopaminergic neurons
3. Maturation and maintenance of dopaminergic neurons
4. Survival of fully differentiated dopaminergic in the neural circuitry

Characterized by the expression of TH the first dopaminergic neurons can be detected in the mouse at E10 when TH expressing cells appear in close proximity to two important signalling centers, the isthmus and the floor plate (Di Porzio et al., 1990), that pattern the neural plate and early neural tube along its dorsoventral and anterior-posterior axes (for review see Wurst and Bally-Cuif, 2001). Explant cultures have revealed two morphogens as molecular entities underlying the activity of these organizers: Sonic hedgehog (Shh) secreted by the floor plate and fibroblast growth factor 8 (FGF8) as mediator of the isthmus (Hynes et al., 1995; Hynes et al., 1995; Ye et al., 1998). While Shh is sufficient to induce dopaminergic phenotypes *in vitro* (Hynes et al., 1995; Wang et al., 1995), *in vivo* it defines the location of dopaminergic neurons in the dorsal-ventral (D-V) axis. FGF8 determines the position along the anterior-posterior (A-P) axis and dopaminergic neurons develop at the intersection within this Cartesian grid (Ye et al., 1998). Shh binds to its receptor patched (Ptc) thereby activating the co-receptor smoothened (Smo) that in its inactive form inhibits Shh signalling. Within the cells activated Smo initiates a signalling cascade leading to the expression of Gli proteins, a family of zinc finger transcription factors (Marti and Bovolenta, 2002). Forced Gli-1 expression in transgenic mice induces ectopic mesencephalic dopaminergic neurons (Hynes et al., 1997), while Gli-2 deficient mice fail to develop mesencephalic dopaminergic neurons (Matise et al., 1998). Once synergistic effects of Shh and FGF8 have induced an early commitment into a dopaminergic fate, regulatory genes are required for initiating and maintaining dopaminergic specific genes.

Specification and maintenance of midbrain dopaminergic neurons has been found to depend on the activity of the transcription factors Lmx1b, Nurr1 and Ptx3. The first transcription factor for correct specification of midbrain dopaminergic neurons that is expressed in the midbrain is Lmx1b. Lmx1b expression commences at E9.5 when

dopamine neuroblasts are still dividing and its expression is maintained in the SN and VTA throughout life (Smidt et al., 2000). Due to its early expression pattern it has been suggested that Lmx1b acts genetically as an upstream activator of the other specifying genes and may be involved in preparing the region for genesis and differentiation of the mesencephalic dopaminergic system (Smidt et al., 2003). Lmx1b deficient mice fail to express Ptx3 but exhibit initial expression of TH and Nurr1, indicating that Ptx3 is not necessary for TH expression (Smidt et al., 2000). However, this set of TH expressing cells is lost during embryonic maturation and it has been concluded that Lmx1b is essential for the proper specification of mesencephalic dopaminergic neurons (Smidt et al., 2003).

Nurr1 and Ptx3 are not expressed in dopamine progenitors and appear only after cell-cycle exit (Smidt et al., 1997; Wallen et al., 1999). Nurr1 is an orphan receptor of the nuclear receptor superfamily widely expressed in the brain of developing and adult rodents (Backman et al., 1999). Nurr1 expression commences at E10.5 in early post-mitotic neurons and continues throughout the lifespan of the cell (Backman et al., 1999). In Nurr1 deficient mice neuroepithelium cells give rise to midbrain dopaminergic neurons of an apparent normal phenotype characterized by the expression of specific markers such as Ptx3 and Lmx1b (Saucedo-Cardenas et al., 1998). However, these dopaminergic precursors fail to differentiate into mature dopaminergic neurons as demonstrated by the lack of TH expression (Zetterstrom et al., 1997; Castillo et al., 1998). The prospective dopaminergic neurons eventually degenerate and disappear resulting in an extensive cell death and the complete lack of mature dopaminergic neurons (Wallen et al., 1999). In line with this the expression of the neurotrophic factor receptor Ret is deregulated in Nurr1 deficient mice (Wallen et al., 2001). Although it has been shown that Nurr1 directly induces TH expression by binding to cis-acting sites within the TH promoter (Iwawaki et al., 2000; Kim et al., 2003), the pure lack of TH expression is probably not sufficient to explain the phenotype of Nurr1-deficient mice since dopaminergic neurons develop normally in TH deficient mice (Zhou and Palmiter, 1995). Although the function of Nurr1 on dopamine neuron differentiation remains unclear, it appears that it is involved in modulating the expression of TH (Sakurada et al., 1999), ret (Wallen et al., 2001) as well as DAT and VMAT (Sacchetti et al., 2001; Smits et al., 2003).

The last factor expressed in this temporal cascade is the homedomain transcription factor Ptx-3 that can be found from E11.5 in rats (Smidt et al., 1997). Its neural distribution is exclusive for midbrain dopaminergic neurons although it can also be found in the developing lens (Semina et al., 1997). Ptx3 responsive elements have been described in the TH promoter (Cazorla et al., 2000; Lebel et al., 2001), but data from Lmb1x deficient mice have already suggested that Ptx3 is not required for TH induction (see above). Indeed TH expression is not affected in the *Aphakia (ak)* mouse that is mutated for the *Ptx3* gene (Semina et al., 2000; Rieger et al., 2001) resulting in a loss of Ptx3 expression in mesencephalic dopaminergic neurons (Smidt et al., 2003). However, anatomical studies revealed that the ventral substantia nigra pars compacta (vSNc) was diminished in these animals (van den Munckhof et al., 2003; Smidt et al., 2003). This selective degeneration of vSNc and about half of the dopaminergic neurons in the VTA was also accompanied by a significant decrease in striatal innervations causing a reduction in spontaneous locomotor activity (van den Munckhof et al., 2003). Since no alteration in the number of dopaminergic neurons was observed at E12.5, but a progressive degeneration at later developmental and postnatal stages caused the observed depletion, it has been suggested that Ptx3 has an involvement in the survival and maintenance of dopaminergic neurons (van den Munckhof et al., 2003).

Maintenance of the dopaminergic phenotype appears to depend on the expression of the homeodomain transcription factors engrailed 1 (En1) and engrailed 2 (En2) that are expressed in proliferating dopamine neuroblasts and post-mitotic dopaminergic neurons (Lin and Rosenthal, 2003). While mutants for En1 or En2 contain SN and VTA, double mutants initially specify these areas but completely lack TH positive cells at birth (Simon et al., 2001) suggesting that survival of dopaminergic neurons requires the *En1* and *En2* genes.

Neurotrophic factors play an important role in the long-term survival of dopaminergic neurons during development and throughout the lifespan of dopamine neurons in adulthood. Most notably for the survival of dopaminergic neurons are neurotrophic factors of the GDNF family comprising GDNF itself (Lin et al., 1993), as well as

neurturin (Kotzbauer et al., 1996), persephin (Milbrandt et al., 1998) and artemin (Baloh et al., 1998). Despite their crucial role for continued survival of dopaminergic neurons and their high potential in therapeutic approaches for the treatment of Parkinson's disease, they seem to have no direct effect on the development of dopaminergic neurons and from this perspective are not discussed further here.

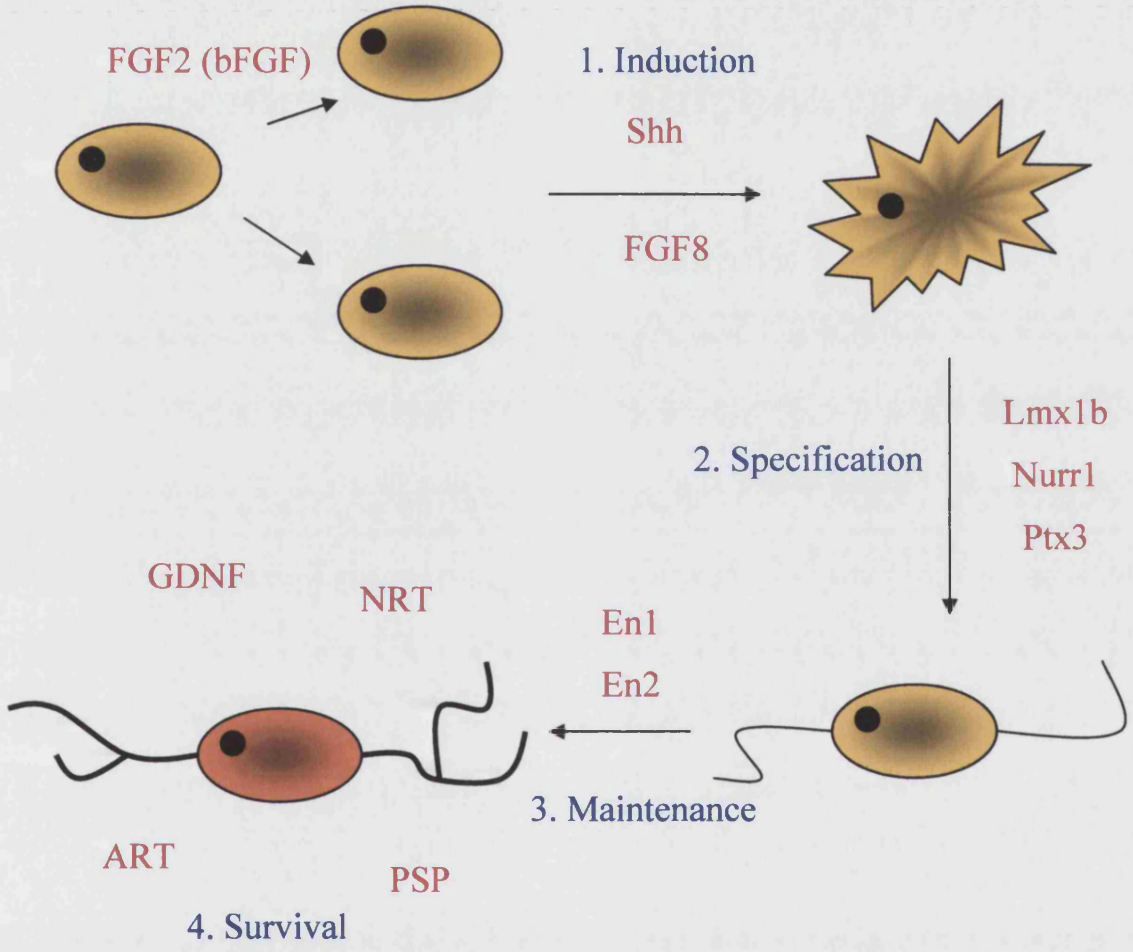


Figure 1-5: Cascade of factors involved in the embryonic development of dopaminergic neurons.

1.5.3 *In vitro* dopamine neuron derivation

As shown in Figure 1-3 various cell sources have been tested for the *in vitro* generation of dopaminergic neurons. The following section focuses on findings for the potential conversion of neural precursors and embryonic stem cells into dopaminergic phenotypes.

Based on the fact that studies of developmental dopaminergic neuron specification have revealed some of the key factors, it would conceptually seem straightforward that these factors are also sufficient for the generation of dopaminergic neurons from neural precursor cells. However, so far such research has contributed little to finding a method for efficiently generating dopaminergic neurons from long term expanded neural precursor cells *in vitro*. Most studies describing the generation of dopaminergic neurons from embryonic or adult derived tissue have in fact used neural progenitor cultures and generally have not considered the long term expansion of generated dopaminergic neurons after multiple passages (Table 1-1).

Progenitor cells isolated from rat E14 ventral mesencephalon and maintained in serum-free conditions give rise to only a small proportion (around 5%) of TH expressing dopaminergic neurons (Cheung et al., 1997). Propagation in the presence of FGF2 can extend dopamine neuron proliferation and differentiation (Bouvier and Mytilineou, 1995) when maintained as primary culture on coated tissue culture plastic ware. In a first approach to increase the yield of dopaminergic neurons, committed dopamine neuroblasts were expanded in FGF2 and differentiated by growth factor withdrawal in the presence of foetal calf serum (FCS) (Studer et al., 1998). Upon transplantation into Parkinsonian rats the precursor cells survived and led to functional recovery. Additional improvement of the basic protocol has also been achieved by reducing the oxygen level to more physiological concentrations (Studer et al., 2000), whereas addition of ascorbic acid promoted dopaminergic differentiation after prolonged expansion (Yan et al., 2001). Similar short-term expansion approaches have also been applied to human midbrain precursors (Sanchez-Pernaute et al., 2001).

However, continuous expansion of the neural progenitor cells as so called neurospheres (neural precursor cells grown as spherical aggregates in suspension cultures) followed by subsequent passaging, rapidly decreases the number of TH expressing cells and efficient generation of dopaminergic neurons from rodent and particularly human tissue has proven difficult (Svendsen and Rosser, 1995; Svendsen et al., 1997; Yan et al., 2001; Storch et al., 2001). In an approach to induce dopaminergic neurons, neurospheres were differentiated into the desired phenotype by a combination of soluble (cytokines, GDNF, FCS and striatal conditioned media) and insoluble (mesencephalic membrane fragments) molecules (Ling et al., 1998; Potter et al., 1999; Carvey et al., 2001). The generated cells survived transplantation into the striatum of hemiparkinsonian rats and ameliorated behavioural impairment (Carvey et al., 2001). Expanded midbrain neurons also exhibit functional properties of dopaminergic neurons *in vitro* (Storch et al., 2003). The same *in vitro* approach combined with low oxygen has also been used to generate dopaminergic neurons from human embryonic mesencephalic precursors (Storch et al., 2001). Although some of these studies report impressive yields of TH expressing cells, there have been concerns about their neuronal identity (Perrier and Studer, 2003). Further, the TH inductive effects depend on the presence of undefined soluble and membrane-bound factors. The observation of TH expressing cells in expanded human mesencephalic neurospheres under defined culture conditions (Riaz et al., 2002) may be the result of surviving dopamine neuroblasts due to the low number of passages performed.

Species	Age	Origin	Expansion	Passaged	Differentiation condition	%TH	Refererence
Rat	E12	VM	plated	No	-	no data	(Bouvier and Mytilineou, 1995)
Rat	E14.5	Mes	suspension	Yes	IL-1, IL-11, LIF, GDNF, mesencephalic membrane fragments, striatal CM, FCS	20-25%	(Ling et al., 1998) (Potter et al., 1999)
Rat	E14.5	VM	suspension	Yes	IL-1, 10%FCS	98%	(Carvey et al., 2001)
Rat	E12	VM	plated	No	10%FCS	14%	(Studer et al., 1998)
Rat	E12	VM	plated	No	3% O_2 only, AA	56%	(Studer et al., 2000)
Human	5-11w	Mes	suspension	Yes	3% O_2, IL-1b, IL-11, LIF, GDNF, striatal co-culture	1%	(Storch et al., 2001)
Mouse	E14	STR	suspension	Yes	B49 CM	5%	(Daadi and Weiss, 1999)
Human	7-8w	Mes	suspension	Yes (3x only)	BDNF, DA, forskolin	60%	(Riaz et al., 2002)
Rat	E14	VM	suspension	No	10%FCS	no data	(Sawamoto et al., 2001)

Table 1-1: *In vitro* differentiation of dopaminergic neurons. Abbreviations: ventral mesencephalon (VM), striatum (STR), conditioned media (CM), ascorbic acid (AA).

In alternative approaches, non-mesencephalic precursor cells (Sakurada et al., 1999; Daadi and Weiss, 1999) and neural stem cell lines (Wagner et al., 1999) have been the target for conversion into a dopaminergic phenotype. Cortical and striatal progenitors produced a rather small number of TH positive cells when expanded in FGF2, but the yield could be increased in the presence of glial cell conditioned media (Daadi and Weiss, 1999). However, the majority of induced TH expressing cells co-labelled with GABA and therefore the phenotype is more similar to that of striatal and olfactory neurons than to that of midbrain dopaminergic neurons. Overexpression of Nurr1 in adult rat hippocampal progenitor cells induced TH expression but did not alter the expression of other midbrain dopaminergic markers (Sakurada et al., 1999) indicating an unspecific action on the TH promoter in neuronal and non-neuronal cell types. Further the study showed that overexpression of Ptx3 in the same system did not cause an increase in the number of TH positive cells, while Shh increased TH expression when it was present during the expansion phase but conversely had repressive effects during the differentiation phase. The presence of FGF8 during the terminal differentiation stages also did not alter TH expression (Sakurada et al., 1999). Interestingly, overexpression of Nurr1 in a mouse neural stem cell, that was previously derived from postnatal cerebellum (Snyder et al., 1992), induced TH expression in combination with as yet unidentified factors derived from type-1 astrocytes (Wagner et al., 1999). The TH positive cells expressed several midbrain dopaminergic markers and released dopamine suggesting that Nurr1 might induce neuronal differentiation in multipotent precursors and provides a competence signal to respond to other soluble factors derived from astrocytes. However, after transplantation the cells survived poorly as compared to fetal mesencephalic neurons indicating that other signals are required for functional integration into the circuitry (Arenas, 2002).

Treatment of primary progenitor cultures from rat and human cortex (Zhou et al., 1994; Zhou et al., 1998; Theofilopoulos et al., 2001) or mouse striatum (Stull and Iacovitti, 2001; Stull et al., 2001) with dopamine and protein kinase-A (PKA) activators (forskolin and isobutylmethylxantine) also induce ectopic expression of TH. An increased number of TH positive cells as well as elevated dopamine release

indicate that BDNF and GDNF have beneficial synergistic effects on the generation of dopaminergic cells from cortical progenitors (Theofilopoulos et al., 2001).

The mechanisms leading to dopamine neuron generation during embryonic development have successfully contributed to improved protocols for the controlled conversion of embryonic stem cells to dopaminergic neurons. Two main strategies have evolved for the efficient generation of dopaminergic neurons.

The first strategy (Lee et al., 2000; Kim et al., 2002) uses the classical approach based on the formation of embryoid bodies in a five step protocol: expansion of undifferentiated ES cells, formation of embryoid bodies, selection of embryoid bodies, expansion of nestin-positive NSCs with FGF2 and finally differentiation into midbrain dopaminergic neurons by withdrawal of FGF2 and in the presence of the morphogens Shh and FGF8 (Lee et al., 2000). Although Shh and FGF8 are not essential for generation of dopaminergic neurons from ES cells, they significantly increase the yield. Further improvements in dopamine neuron yield were obtained in ES cell lines genetically engineered to overexpress Nurr1 (Kim et al., 2002; Chung et al., 2002). The disadvantage of this traditional protocol is the extended *in vitro* culture periods required, and recently a more rapid protocol for the selective generation of neural cell types including dopaminergic neurons has been developed (Barberi et al., 2003). The dopaminergic neurons derived from this protocol express several midbrain specific dopaminergic markers, release dopamine, exert electrophysiological properties of dopaminergic neurons and most notably survive, integrate and ameliorate impairment after intrastriatal grafting into hemiparkinsonian rats (Kim et al., 2002). Interestingly, some of the generated neurons are serotonergic, which are normally induced by Shh and FGF8 in the hindbrain region (Ye et al., 1998), suggesting that by mimicking the midbrain-hindbrain expression pattern *in vitro*, a similar cellular diversity can be induced to that of the isthmus and floor plate *in vivo*. Dopaminergic and serotonergic neurons can also be generated with clonal ES cell lines derived from nuclear transplantation of adult donor cells (Wakayama et al., 2001). The protocols have also been the basis for generation of ES cell derived dopamine neurons from non human-primates (Perrier and Studer, 2003), but a direct adaptation to generate dopaminergic neurons from human ES cells has proved difficult (Zhang SC, personal communication). It has recently been

suggested that BDNF and TGFα are inductive for the production of dopaminergic cells from human ES cells (Park et al., 2004).

The other reported strategy relies on the inductive activity of co-culture with a bone marrow-derived stromal cell line, so-called stromal cell derived inducing activity (SDIA) (Kawasaki et al., 2000; Kawasaki et al., 2002). The method rapidly induces dopaminergic neurons independent of morphogenetic factors such as Shh or FGF8. However, the molecular identity of the factors within the SDIA has remained unknown. Interestingly, the inductive activity is retained after fixation or without any physical contact but could not be exerted by conditioned media (Kawasaki et al., 2000). The prospective dopaminergic neurons produced, survived and integrated after grafting into 6-OHDA depleted striatum. However, functional recovery has not yet been demonstrated. The midbrain fate can be altered by sequential exposure of mouse ES cells to SDIA, retinoic acid (as caudalizing inducer) and Shh (here as ventralising factor) to differentiation into a spinal motor neuronal cell type (Wichterle et al., 2002) suggesting that SDIA supports a general neural promoting factor rather than being a specific dopaminergic neuron inducer.

The major drawback of ES cell derived dopaminergic neurons is, apart from ethical concerns, the possible generation of teratomas upon transplantation. With 20% of animals received ES transplants developing teratomas (Bjorklund et al., 2002) this is a serious safety issue to be overcome before clinical trials could be considered.

Summarising the previous studies, it has been shown that although neural progenitors derived from the developing ventral mesencephalon can be expanded for a short time, their long term expansion and controlled induction into a dopaminergic phenotype under defined conditions has proven difficult. Morphogenetic and inductive factors which are required for embryonic development *in vivo* also have effects on the generation of dopaminergic neurons from ES cells. However, they have not been demonstrated to sufficiently induce a dopamine phenotype in foetal or adult precursor cells. By definition all stem cells are multipotent, but practically, the distinct stem cell systems exhibit intriguing differences in their responsiveness to developmentally relevant signals. This leads to questions concerning the identity of different stem cells. Do stem cells change throughout development, and are neuroepethilium stem cells the

same as a prospective adult stem cell? The inability of long-term expanded neurospheres to give rise to dopaminergic neurons is described as the "dopamine problem" (Svendsen, personal communication), and it will be the challenge of future studies to identify factors that are capable of inducing the desired neural phenotype from neural stem cells of this type.

1.6 Viral vectors for gene delivery to neural precursor cells

With the discovery that neural stem cells exist in the mammalian brain and with the knowledge that we have acquired over the past two decades about their isolation and propagation, together with advances in molecular biology, virology and the understanding of neurodevelopment, the genetic manipulation of NSCs for both basic research and therapeutic applications has become feasible. The introduction of exogenous genetic material into neural stem cells can accomplish different purposes: to label and monitor neural stem cells, to guide their development into a specific cell type, to release therapeutic factors, to target specific genes or to study human diseases. The commonly used viral vectors for gene targeting to neural stem cells are adenoviruses, adeno-associated viruses, retroviruses, and lentiviruses (Asahara et al., 2000). However, it is the aim of the study that defines which vector system is most appropriate. If for instance the aim is to label exclusively dividing cells, a method frequently used to monitor stem cells *in vivo*, a vector is required that only transduces proliferating cells and the vector choice is limited to retroviruses. In cases that only a transient transgene expression over a limited number of cell divisions is desired, non-regulated integrating viral vectors are inappropriate and non-integrative vectors such as adenoviruses or HSV-1 should be considered. For the purpose of gene therapeutic applications, integrative vectors have another major disadvantage. Due to random integration into the cellular genome, proto-oncogenes may be activated causing cancer. Recent gene therapy clinical trials for severe combined immunodeficiencies (SCID) have shown that this is indeed a very serious problem. Here, bone-marrow stem cells were isolated from the patients, infected with retroviruses delivering the therapeutic gene to replace the genetic deficit and were then re-implanted into the patient, where they were hoped to multiply to normal immune cells to reconstitute the patients immune function. Although the majority of patients benefited from the treatment, some developed leukaemia-like conditions (Hacein-Bey-Abina et al., 2003). Genetic analysis showed that the retroviral vectors had integrated into, and activated, an oncogene called *LMO2* that is associated with childhood leukaemia. Further development of safer vectors is needed to avoid such risks in future trials.

General requirements that can be defined for all vector systems for gene delivery to neural stem cells are high transduction efficiency, low viral cytotoxicity and no effects on the intrinsic cellular function and stem cell character of the transduced neural precursor cell. Other properties to consider are the production of the vector as it should be easy to be generated and grown to high titre, it should be immunologically inert to avoid immunological clearance, and have the appropriate size capacity to accommodate the foreign genetic material.

The following section aims to give an overview of the most commonly used viral vectors for gene delivery to neural stem cells. Non-viral vectors give too low transduction efficiency to neural stem cells and will not therefore be further considered.

1.6.1 Adenoviruses

Adenoviruses (Ads) are non-enveloped double stranded DNA viruses with genomes of approximately 36kb in length, which usually remain as episomal elements within the host cell nucleus. In humans there are 50 different serotypes, which are classified into six groups termed A-F (reviewed in Horwitz, 2001). Members of the subgroup C are generally thought to be the most suitable for gene therapy vectors, due to low pathogenicity, and of this subgroup, Ad1, Ad2, Ad5 and Ad6 are regarded as endemic within the population (Murphy, 1999).

The genome is functionally divided into two major regions, termed late and early, according to their time of transcription after infection. These two regions contain overlapping transcriptional units and in total encode for 50 polypeptides (Kay et al., 2001). Ads exert a broad cell tropism and can infect both dividing and non-dividing cells (Benihoud et al., 1999). They can easily be genetically modified and purified to high titres (Lundstrom, 2003).

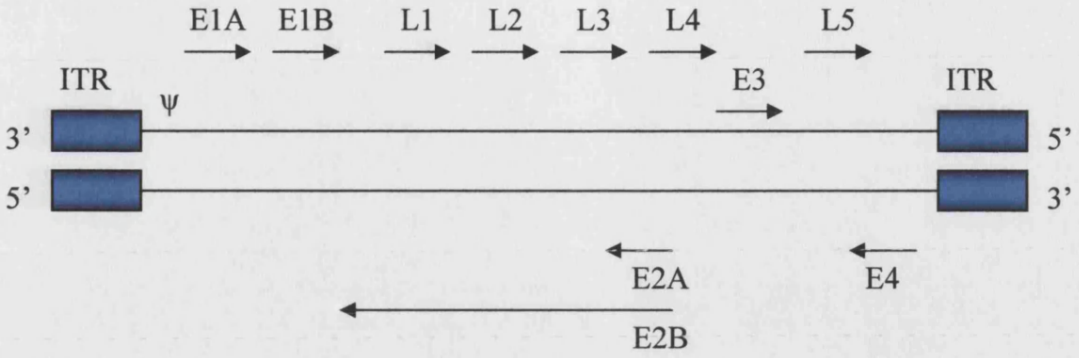


Figure 1-6: Schematic of the adenoviral genome. Ψ = packaging signal, E = early gene expression, L = late gene expression.

First-generation recombinant Ads are generated by deleting the parts of the virus genome that are required for replication (E1 region), and replacing them with a transgene expression cassette. In addition to express the transgenes of interest, some of these vectors still produce low-level expression of capsid and other virus proteins. This residual viral gene expression is responsible for transient transgene expression since early cytokine responses, followed by antigen-dependent immune responses lead to a cell-mediated destruction of transduced cells. Newer second- and third-generation vectors with many of the genes deleted, have demonstrated significant improvements (Schiedner et al., 1998). Gutless vectors, in which all adenovirus genomic sequences have been deleted except for the non-coding inverted terminal repeats and packaging signal, have an increased transgene capacity (up to 36kb total), diminished immune activation, and the ability to confer long-term gene expression (reviewed by Davidson and Breakefield, 2003). The helper virus contains all of the necessary Ad replication and packaging signals that act *in trans* on a vector containing the necessary *cis* acting elements and the foreign gene. The helper virus is unable to package itself but aids the production of recombinant virions that lack all the Ad genes except for the *cis* acting elements. However, production of these gutless vectors require a helper-packaging virus, is labour intense and difficult to scale up to pharmaceutical levels. Moreover, current methods of vector preparations still contain helper virus contamination.

Adenoviruses efficiently transduce primary rodent (Caillaud et al., 1993) and human neural progenitor cells (Sabate et al., 1995; Corti et al., 1999), as well as neural

precursor cells generated from the developing and adult rodent CNS (Gage et al., 1995; Hughes et al., 2002; Falk et al., 2002) and the developing human brain (Keyoung et al., 2001). Other studies also showed the efficient gene delivery to postmitotic neurons and embryonic stem cells with low cytotoxicity using gutless vectors compared to first generation vectors (Cregan et al., 2000; Smith-Arica et al., 2003). While neuronal physiology is disrupted upon infection with first generation viruses, normal physiological responses were seen with helper-dependent Ads with adverse effects only at high titres (Cregan et al., 2000). Efficient gene delivery has also been demonstrated to other neural cell types, such as astrocytes (Smith et al., 1996) and oligodendrocytes (Le Gal et al., 1993), as well as ependymal cells (Bajocchi et al., 1993), and microglia (Bhat and Fan, 2002). Targeted gene expression to neurons has been achieved by construction of Ads with neuron-specific enolase (NSE) promoters (Navarro et al., 1999).

Infection of EGF expanded neurospheres with adenovirus expressing EGFP under an RSV promoter resulted in a reasonable transgene expression 16hr post transduction and could be maintained for one month *in vitro* (Hughes et al., 2002). However, adenoviral transduction induced a premature differentiation of progenitors into GFAP positive astrocytes, even when maintained in the presence of the mitogen EGF. This occasional differentiation is accompanied by a decrease in nestin expression. It was thaught that this premature differentiation may result from G1 progression due to adenoviral gene expression (Hughes et al., 2002). Removal of the growth factor induced differentiation of the transduced population into predominantly astrocytes, only about 2% of the infected cells being positive for the neuronal marker MAP2. Altered differentiation has also been observed upon adenoviral transduction of adult mouse neural precursor cells (Falk et al., 2002). Precursors derived from the ventricle were expanded as neurospheres in the presence of EGF and transduced with adenovirus expressing GFP under CMV promoter control resulting in a gene delivery efficiency of about 68%[1](Falk et al., 2002). However, this phenomenon was not observed when transgene expression was targeted to a selective population of neural stem cells derived from human fetal tissue expanded as neurospheres in EGF and

[1] No m.o.i. provided in this reference.

bFGF (Keyoung et al., 2001). This study uses adenoviruses expressing GFP under cell specific promoters to selectively label a subpopulation of uncommitted, dividing progenitor cells. Using EGFP under the nestin enhancer, a primitive population of nestin positive cells was selected of which only 3% were positive for βIII-tubulin and 9% for GFAP. No predifferentiated progenitors were noted when hGFP was driven by the musashi promoter (Keyoung et al., 2001), an RNA binding protein expressed by neural progenitors (Sakakibara et al., 1996). This discrepancy may be the result of species differences or reflect the diversity of progenitor subpopulations within neurospheres. The premature differentiation of virally infected cells described by Hughes *et al.* may only occur in a subpopulation of already fate committed progenitor cells, but not in uncommitted precursor cells as shown by Keyoung *et al.* Further Keyoung *et al.* showed by retroviral lineage analysis that the selected adenoviral infected precursor cells retain their multipotent character even at multiple passages (Keyoung et al., 2001).

A strong and controlled transgene expression could also been observed upon transduction of human progenitors expanded in bFGF with an adenovirus expressing tyrosine hydroxylase under a CMV promoter (Corti et al., 1999). The adenoviral construct used in this study takes advantage of a regulated transgene expression using the tet-off system for a controlled expression *in vitro* and *in vivo* (Corti et al., 1999).

Adenoviruses also deliver efficiently to endogenous stem cells in the SVZ as demonstrated by injections into the lateral ventricle of mice (Bajocchi et al., 1993; Yoon et al., 1996) and rats (Benraiss et al., 2001) without any significant cytotoxic effects occurring. A strong transgene expression in the lateral wall of the SVZ was observed for at least two months after injection of $1.2x10^6$PFU of the recombinant virus driving LacZ under a CMV promoter. The transduced progenitor cells retain their capacity to proliferate and migrate along the rostral migratory stream to the olfactory bulb where they differentiate into granular and periglomerular interneurons[2] (Yoon et al., 1996). As the migration of the neuroblast occurs as a result of a continuous event of cell divisions (Lois and Alvarez-Buylla, 1994), the high number

[2] Only characterized by morphology and not by staining with cell specific markers.

of transduced progenitors in the olfactory bulb is surprising due to the non-integrative nature of the adenovirus. One possible explanation is a continuous arrival of transduced neuroblasts from the SVZ, which is confirmed by the observation that the number of transduced cells in the olfactory bulb increases over time and a time dependent maturation of the neuroblasts into differentiated neurons with a distinct mature morphology (Yoon et al., 1996). Intraventricular injections of adenoviruses expressing hGFP under a CMV into adult rats also confirmed a high efficiency of gene delivery restricted to the ependymal and subependymal layer of the SVZ over a time course of at least three weeks (Benraiss et al., 2001).

1.6.2 Adeno-associated viruses

Adeno-associated viruses (AAV) is a member of the human parvovirus family, small single-stranded DNA viruses that require a helper virus, such as adenovirus or herpes-simplex virus, for replication (for review see Okada et al., 2002). Although the majority of the population is seropositive for AAV, no known disease is associated with AAV infection, making it an ideal candidate for gene therapy. There are six different human serotypes known that have shown different expression patterns because of differences in cell entry and intracellular activities. Wild type AAV preferentially integrates into a specific locus on human chromosome 19, facilitated by the *rep* gene products (Samulski, 1993), but this property is lost in vectors due to the absence of the *rep* gene. AAV vectors infect a wide variety of cell types that are both dividing and non-dividing and vectors have been shown to remain episomally (Duan et al., 1999) or to integrate randomly (Miao et al., 2000).

The virus contains two genes, *rep* and *cap*, encoding polypeptides important for replication and encapsidation, respectively.

Figure 1-7: Schematic of the AAV genome. Ψ = packaging signal.

Creation of AAV vectors usually involves the replacement of the *rep* and *cap* genes with transgenes. These two genes can be supplied *in trans* with only the inverted terminal repeats (ITRs) required *in cis* for viral replication. Therapeutic genes can therefore be inserted between the two ITRs, and vectors produced by co-transfection with plasmids encoding *rep* and *cap* and subsequent infection with a helper virus (Rolling and Samulski, 1995). Methods have been described using a single herpes simplex virus providing both helper virus function and *rep/cap* genes in one single simple step, which avoids transfection (Booth et al., 2004). Since transfection is difficult to scale up, this has significant advantages in generation of large scale virus preparations. The main disadvantage concerning these vectors are related to the small cloning capacity (about 4.8kb), however, this can be overcome by infection of cells simultaneously with two AAV vectors, which can recombine to generate a larger genome (Duan et al., 2003).

Primary mesencephalic progenitor cells derived from E14 time-mated rats can be infected with AAV at high multiplicity (m.o.i. of 25,000) without obvious cytotoxic effects (Fan et al., 1998). Gene delivery to mesencephalic progenitors *in vitro* is rather low but can be enhanced by using HSV/AAV hybrid vectors (Costantini et al., 1999) containing elements of HSV-1 amplicons and recombinant AAV vectors (Johnston et al., 1997). However, long term gene expression *in vitro* and *in vivo* was only possible with hybrid vectors containing the AAV *rep* gene (Costantini et al., 1999).

AAV2 gave unsatisfying gene delivery to mouse neural progenitors expanded as neurospheres with EGF (0.01% reporter gene positive cells at an m.o.i. of 2), while no reporter gene expression was observed with serotypes 4 and 5 (Hughes et al., 2002) and it has been suggested that this may be due to the lack of required receptors or extracellular matrix proteins interfering with viral uptake (Hughes et al., 2002). However, other studies have shown that human derived forebrain progenitor cells expanded in EGF could efficiently be infected with recombinant AAV2 expressing EGFP with an onset of expression one day after infection (Wu et al., 2002). Significant differences between the vectors are only the choice of promoter, Hughes

et al. are using an RSV promoter while Wu *et al.* are using a CAG promoter[3], which might explain this controversial result. Mouse and human ES stem cells infected with AAV expressing the reporter gene under an RSV promoter demonstrated moderate transgene expression when infected with AAV2, but no expression upon infection with AAV4 or AAV5 (Smith-Arica et al., 2003). Furthermore, reporter gene expression disappeared after differentiation of the AAV2 infected ES cells. On the other hand, ES cells differentiated before transduction retained susceptibility to infection with all three AAV serotypes and it has been suggested that the absence of infection in undifferentiated ES cells may reflect the lack of the required receptors known to be involved in their attachment and internalization (O- and N-linked sialic acid; (Kaludov et al., 2001). AAV2 infected human neural precursor cells have decreased proliferation rates but retain their capacity to differentiate into neurons and glia, and furthermore survive and express the reporter gene after transplantation (Wu et al., 2002).

In vivo AAV serotype 2 infects neurons preferentially (Bartlett et al., 1998), apparently through the interaction of AAV2 capsid proteins with heparan sulphate proteoglycan (HSPG) moieties on the cell surface (Davidson and Breakefield, 2003). High level transgene expression in neuronal cell types depends on the choice of promoter (Haberman and McCown, 2002), mammalian promoters generally achieving more sustained transgene expression than viral promoters. Other AAV serotypes have also been found useful in brain-directed gene transfer, showing that AAV4 transduces ependymal cells more efficiently than AAV2 and AAV5 infects neurons and astrocytes *in vivo* unlike AAV2 which only infects neurons (Davidson et al., 2000). Differences between serotypes are most likely due to the fact that the capsid proteins use different cellular receptors for entry. Intraventricular injections of AAV2 results in dispersed gene delivery throughout the CNS (Passini and Wolfe, 2001), however, many brain areas remained poorly transduced. In contrast, injection of AAV1 into the lateral ventricles of mice produced a robust and extensive pattern of transduction with transgene expression in the ventricle wall of the SVZ-ependymal region throughout

[3] The CAG promoter is a chimeric promoter that consists of a CMV immediate-early enhancer, a chick β-actin promoter, a chimeric intron, exon 1 and a part of exon 2 of a rabbit β-globin gene and has been shown to have a higher activity than the CMV promoter in mammalian cells (Wu et al., 2002).

the entire ventricle system and the olfactory bulb (Passini et al., 2003). Immunohistochemical staining revealed that the majority of infected cells co-labelled with neuronal marker, but not with astrocyte- or oligodendrocyte-specific markers (Passini et al., 2003) suggesting that neural precursor cells were not targeted. AAV vectors of different serotypes also efficiently and stably deliver reporter gene expression to retinal progenitor cells *in vivo* (Surace et al., 2003).

Recently it has been shown that AAV can successfully be used as gene targeting vectors in adult human stem cells derived from individuals with osteogenesis imperfecta (OI) (Chamberlain et al., 2004). OI is caused by mutations in collagen genes that result in severe skeletal abnormalities. Mesenchymal stem cells (MSCs) from OI patients were isolated and infected with AAV gene targeting vectors that disrupted the mutated gene in the MSCs. AAV gene targeting improved collagen processing, stability and structure. Furthermore, targeted MSCs retained the multipotent capacity to differentiate into bone and fat cells demonstrating that gene targeting is possible in nonembryonic human stem cells (Chamberlain et al., 2004).

1.6.3 Retroviruses

The utilization of viruses as efficient vehicles to transfer recombinant DNA into eukaryotic cells first used retrovirus vectors (Cepko et al., 1984). Retroviruses are lipid-enveloped single-stranded RNA viruses with genomes of 7 to 11 kilobases (reviewed by Kay et al., 2001). The retrovirus family includes mammalian and C-type retroviruses, lentiviruses and spumaviruses. The majority of clinical trials to date have used vectors based on the murine leukaemia virus (MLV) (reviewed in Robbins et al., 1998).

Productive transduction by retroviral vectors is strictly dependent on target cell mitosis shortly after entry (Miller et al., 1990). Because only a fraction of cells pass through mitosis at any given time, this severely limits the applications of retroviral vectors in gene therapy. Following entry into target cells, the RNA genome is retro-transcribed into linear double-stranded DNA and integrated into the cell chromatin. Integration does not guarantee stable expression of the transduced gene, but it is an

effective way to maintain the genetic information in a self-renewing cell source, such as neural precursor cells.

The retrovirus genome contains two long terminal repeat (LTR) sequences flanking the three viral genes *gag*, *pol*, *env* and the packaging signal (reviewed by Kay et al., 2001). The viral genes encode for structural proteins, nucleic-acid polymerases/integrases and surface glycoproteins. The viral envelope glycoprotein determines the host range of retroviral particles through the interaction with receptors on target cells. To overcome limitations of host cell tropism, retrovirus vectors have been pseudotyped with envelope proteins from other viruses such as the G glycoprotein from vesicular stomatitis virus (VSV) (Burns et al., 1993). VSV G pseudotyped retroviruses are less labile, can be concentrated to higher titres and have a much broader host range.

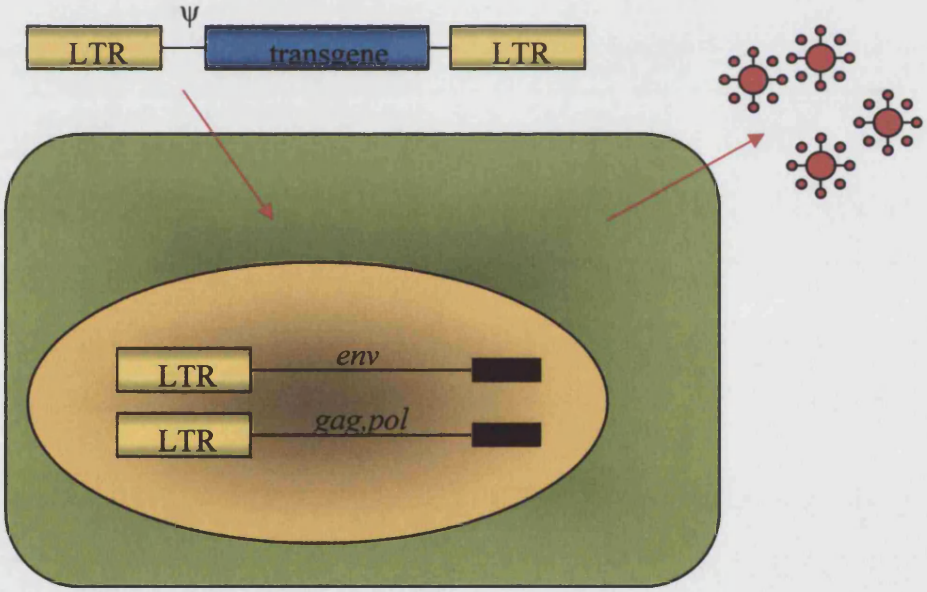


Figure 1-8: Generation of retrovirus based vectors.

Retroviral vectors are constructed by removal of all viral genes replaced by the transgene of interest (up to 8kb of foreign genetic material) and flanked by the remaining *cis* regulatory elements (Robbins et al., 1998). Packaging cell lines expressing the viral genes *gap*, *pol*, *env* are either stably or transiently transfected with the recombinant retroviral construct and because only the vector contains the packaging sequence and the two LTRs, only vector RNA is packaged into virions.

Separation of the *gag/pol* genes from the *env* gene is required to avoid recombination resulting in the production of replication competent retroviruses (Donahue et al., 1992).

Because of their inability to deliver genes to non-dividing cells, retroviral vectors have found only limited application as gene therapeutic vectors in the CNS. However, this feature has made them a unique tool to label the rare population of dividing neural stem/precursor cells in the adult brain and follow their differentiation into mature neural cell types and functional integration in the circuitry in many studies (for example Fields-Berry et al., 1992; Doetsch et al., 1999; van Praag et al., 2002; Suzuki and Goldman, 2003). The retro-viral gene transfer method has become a reliable way to monitor migration and neuronal differentiation of precursor cells *in situ* (Rakic, 2002), even in live time-lapse videomicroscopy (Suzuki and Goldman, 2003). Furthermore, these vectors have extensively been used for *ex vivo* infection of cultured neural precursor cells followed by transplantation (Flax et al., 1998; Torchiana et al., 1998; Liu et al., 1999; Meng et al., 2003) and for clonal analysis in brain development (Turner et al., 1990; Fields-Berry et al., 1992; Williams and Price, 1995). Although retroviral vectors have found application in many gene therapeutic trials, the fact that integration occurs randomly, and thus may have adverse effects on normal cellular function (see above), requires the further development of safer vectors.

Originally with the aim of infecting embryonic stem cells, a murine embryonic stem cell virus (MESV) has been developed from a retroviral mutant by manipulation of the 5' untranslated region that abolished viral gene expression in ES cells (Grez et al., 1990). This MESV was further altered by removal of all potential start sites of translation in the 5' leader and replacement of the *gag* gene sequences with a constitutive transport element from aevian leukaemia virus to promote nuclear export of viral RNA (Owens et al., 2002). The generated vectors have been referred to as MESV2 and were demonstrated to infect rat hippocampal precursors cells *in vitro* that retained their ability to differentiate into neurons and astrocytes upon differentiation (Owens et al., 2002).

1.6.4 Lentiviruses

Unlike retroviruses, lentiviruses rely on the active transport of the preintegration complex through the target cell nucleopore (Lewis et al., 1992), a feature that enables them to transduce both dividing and non-dividing cells. The vector gives long-term expression through chromosomal integration. Pseudotyping with VSV-G glycoprotein has expanded the vector tropism (Naldini et al., 1996) and made lentiviral vectors a promising tool for gene therapy (Kay et al., 2001).

The lentiviral genome is more complex than that of retroviruses and contains two additional regulatory genes, *tat* and *rev*, essential for the expression of the genome and a variable set of accessory genes (Naldini, 1998). The accessory genes appear to have no obvious function *in vitro*, but are required for full virulence and pathogenesis *in vivo* and do not need to be complemented in culture (Naldini et al., 1996). The generation of "minimal" packaging constructs have been adopted to increase vector biosafety (Dull et al., 1998). The fact that many of the lentiviruses used in gene therapy are based on the human immunodeficiency virus (HIV) has raised safety concerns and lead to the development of self-inactivating transfer vectors (Zufferey et al., 1998; Miyoshi et al., 1998). The vectors contain a deletion in the downstream LTR that after transduction results in the transcriptional inactivation of the upstream LTR and thus diminishes the risk of vector mobilization and recombination (Bukovsky et al., 1999).

Lentiviral gene delivery to the CNS causes a minimal inflammatory response and produces long-term transgene expression when introduced into the brain parenchyma or ventricles (Naldini et al., 1996; Brooks et al., 2002). Lentiviral vectors have been used for the efficient transduction of rodent (Ostenfeld et al., 2002) and human (Englund et al., 2000) neural precursor cells grown as free floating neurospheres. Gene delivery rates were between 75% (Ostenfeld et al., 2002) and 90% (Englund et al., 2000) at an m.o.i. of 5. No obvious cytotoxic effects were observed (Buchet et al., 2002), but lentiviral transduction slowed down cell proliferation in human precursor cells (Englund et al., 2000). After transplantation into the striatum of rats the infected precursor cells survive and express significant amounts of the transgene (Englund et

al., 2000; Ostenfeld et al., 2002). The transduced precursors retain the ability to differentiate into different neural cell types after grafting *in vivo* (Buchet et al., 2002). Intraventricular injections of retroviral and lentiviral vectors result in an inefficient transduction of cells in the SVZ-ependymal region (Johansson et al., 1999; Falk et al., 2002), and thus are not suitable for extensive delivery of therapeutic genes to this region.

1.7 Herpes simplex virus

Members of the *Herpesviridae* form a large and diverse family that comprises *alpha-*, *beta-*, and *gammaherpesviruses*. Herpes simplex viruses (HSV) belong to the family of *alphaviruses* (Roizman and Pellet, 2001) and are among the most intensively studied of all viruses. HSV-1 and HSV-2 are natural pathogens in humans, and are responsible for mucocutaneous lesions, but can also rarely cause meningitis and encephalitis (Spear and Longnecker, 2003). HSV-1 is endemic within the human population, with 80% of individuals testing positive for antibodies to HSV-1, and commonly establishes latent infections in neurons with the potential of reactivation to cause recurrent or new episodes of the disease (Roizman and Knipe, 2001).

1.7.1 Potential of HSV-1 as a gene delivery vector for neural precursor cells

So far HSV-1 has received attention as a gene delivery vector for gene therapeutic applications for several reasons (reviewed in Glorioso et al., 1995; Burton et al., 2001; Davidson and Breakefield, 2003):

- **Broad host cell range including infection of dividing and non-dividing cells.** Although HSV-1 is neurotrophic *in vivo* it efficiently infects a broad range of cell types *in vitro* and *in vivo*. Wildtype HSV-1 is cytotoxic to the infected cell, but by deleting multiple viral genes replication deficient, non-cytotoxic vectors can be generated that persist in a latency like state (Samaniego et al., 1998) in neurons and non-neuronal tissue (Wolfe et al., 1999). Repeated vector administration is possible even in immune hosts.

- **Establishment of latency gives the potential for life-long sustained transgene expression.** During latency HSV-1 remains episomal and infected neurons function normally and are not rejected by the host immune system. Furthermore, the latency active promoters can be exploited in heterologous expression cassettes for long-term transgene expression (Palmer et al., 2000; Lilley et al., 2001).

- **HSV-1 can accommodate a large amount of foreign genetic material.** The genome contains a large number of accessible genes that are not required for viral growth in culture and essential genes that can be provided *in trans*. This feature makes HSV-1 a large capacity vector capable of harbouring at least 30kb of foreign gene sequences encompassing large single genes or multiple gene cassettes that may be coordinatively or simultaneously expressed (Krisky et al., 1998).

- **Recombinant HSV-1 is easy to manipulate and propagate.** Highly replication-deficient viruses can be grown on cell lines providing the deleted genes *in trans* in titres of up to 10^9 plaque forming units per ml (PFU/ml).

Several features make HSV-1 particularly interesting for the transduction of stem cells. Due to its capability of incorporating large DNA inserts, it may be possible to express multiple genes to induce differentiation to a specific cell lineage. As the viral genome persists episomally without disturbing host cell metabolism this avoids the risk of integration related mutagenesis and the potential activation of proto-oncogenes (Burton et al., 2001). However, because of the non-integrating nature of HSV-1 vectors, transgene expression will only be maintained in the transduced stem cell or progenitor cell over a small number of cell divisions and will not occur in cells found many cell divisions away from the transduction event. For the induction of a specific phenotype, requiring only a transient expression of the differentiating signal, this may be advantageous.

On the other hand, due to its non-integrative nature, HSV-1 is not suitable for delivery to neural stem cells with the purpose of continuously expanding the transduced precursor cell population. For this purpose integrative vectors are more appropriate.

HSV-1 as a vector system for stem cells has primarily been described to hematopoietic stem cells (reviewed in Burton et al., 2001; Wolfe et al., 2004), showing efficient transduction of progenitor cells derived from human bone marrow (Dilloo et al., 1997) and human blood (Coffin et al., 1998). Although amplicon based

HSV-1 viruses have been used to efficiently transduce embryonic stem cells (Anderson et al., 2001; Vicario and Schimmang, 2003), no thorough study has been described using HSV-1 as a gene delivery vector to neural stem cells of the central nervous system. Therefore, it will be challenging to study the potential application of HSV in other stem cell lineages, such as neural stem cells.

1.7.2 Fundamental biology of HSV-1

1.7.2.1 The HSV-1 genome and structure of the virion

HSV-1 is a double-stranded, enveloped DNA virus. Its genome size is about 152 kb and encodes more than 80 polypeptides (Roizman and Knipe, 2001). The HSV genome is arranged as long and short unique segments (U_L and U_S) that are flanked by repeated regions, a, b and c (McGeoch et al., 1986; Perry and McGeoch, 1988; McGeoch et al., 1988). About half of the viral genes are non-essential and therefore not required for viral replication in a permissive tissue culture environment. Deletion or replacement of these genes does not require substitution *in trans* for virus growth, but they may play a role in specific virus-host interaction *in vivo* (Krisky et al., 1998).

The natural route of HSV-1 uptake is by infection of skin epithelial cells, where upon the virus undergoes a cycle of lytic gene expression and replication (Roizman and Knipe, 2001). Following lysis the released viral particles infect sensory nerve terminals and are retrogradely transported to the neuronal cell body. From there on, the virus can either undergo a further round of replication or alternatively, establish a latent infection. During latency the viral genome can persist episomally for the lifetime of the cell or be reactivated to re-enter the lytic cycle followed by anterograde axonal transport to skin or mucosal surfaces where it results in the production of infectious particles at or near the original site of the primary infection.

The mature HSV-1 virion contains four components: the core of double-stranded viral DNA within an icosahedral capsid, surrounded by a protein-rich layer, the tegument, which is finally embedded within the lipid envelope (Roizman and Knipe, 2001).

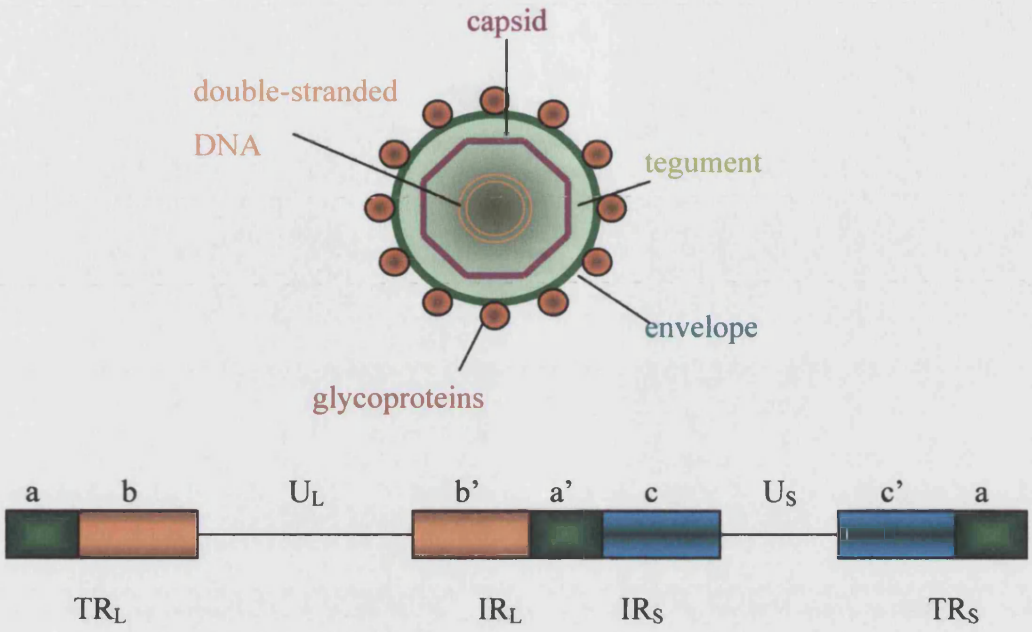


Figure 1-9: The HSV-1 virion and genome.

1.7.2.2 Virus entry

The initial steps of viral infection include the interaction between virion and target cells (attachment) followed by incorporation of the virion into the cell (entry). This is facilitated by interaction between the viral envelope containing at least 11 glycoproteins (named gB to gM) and receptors on the cell surface of the target cell.

Although different cell surface molecules can serve as receptors for viral attachment, it has been established that the initial contact of HSV-1 with cells is mediated by binding to glycoaminoglycans, usually heparan sulphate (HS), on cell surface proteoglycans (Shukla and Spear, 2001). HS are linear polysaccharides which are ubiquitously biosynthesized as a proteoglycan in mammalian cells (reviewed by Rabenstein, 2002) including neural precursor cells (reviewed by Ford-Perriss et al., 2002). The particular role HS plays as portal of entry has been demonstrated in several experimental rationales: Heparinase treated cells and prevention of HS biosynthesis significantly reduce virus binding and infectivity (WuDunn and Spear, 1989; Shieh et al., 1992); soluble heparin and forms of HS inhibit viral infection by binding of viral particles (Nahmias and Kibrick, 1964; WuDunn and Spear, 1989);

soluble forms of glycoprotein B (gB) and glycoprotein C (gC) bind heparin and HS (Svennerholm et al., 1991; Tal-Singer et al., 1995; Williams and Straus, 1997; Trybala et al., 2000) and competitively inhibit attachment to cells (Svennerholm et al., 1991; Tal-Singer et al., 1995). Therefore, binding of the virion to HS appears to be mediated by the viral glycoprotein gB and gC, although gC is dispensable but significantly increases the efficiency of virus binding (Spear and Longnecker, 2003). The binding to HS is not essential for virus infection, but the presence of HS increases the efficiency of viral entry, probably by concentrating virus on the cell surface for subsequent interaction with the viral entry receptors (Spear and Longnecker, 2003). While initial studies identified heparan sulphate as receptor for HSV-1 attachment in endothelial cells (Herold et al., 1994), Immergluck *et al.* showed that heparin sulphate is also required for HSV-1 entry into primary neural progenitor cells derived from the developing chick cortex (Immergluck et al., 1998). Although in endothelial cells the interaction with heparan sulphate is predominantly facilitated by HSV-1 gC as shown in several studies using either neutralising antibodies for gC (Fuller and Spear, 1985; Svennerholm et al., 1991) or mutational analysis of gC (Campadelli-Fiume et al., 1990; Herold et al., 1991; Mardberg et al., 2001), gC has no significant effect on initial virus binding in neural progenitor cells (Immergluck et al., 1998), and gB may provide functional redundancy in neural cell types.

However, HS is not the only cell surface receptor mediating virus attachment. Another factor, noteworthy here due to its role in infecting neural stem cells, is basic fibroblast growth factor receptor. Although this receptor plays a role in virus attachment (Baird et al., 1990), its originally suggested essential function as the entry receptor (Kaner et al., 1990) has later been disproved (Mirda et al., 1992; Muggeridge et al., 1992).

The initial and reversible binding to HS is followed by a cascade of irreversible receptor-ligand interactions at the cell surface that trigger fusion between viral envelope and the plasma membrane and ultimatively results in virus entry (Spear and Longnecker, 2003). Four of the envelope glycoproteins are absolutely essential for entry of HSV-1, namely gB, gD, gH, and gL (reviewed by Spear and Longnecker, 2003). Three classes of HSV-1 entry receptors have been identified on the surface of cells: a member of the TNF-receptor family named HVEM (HveA) (Montgomery et

al., 1996), members of the immunoglobulin family referred to as nectin-1 (HveC) (Geraghty et al., 1998) and nectin-2 (HveB) (Warner et al., 1998), and a modified form of HS that generates binding sites for gD (Shukla et al., 1999). Any of these cell surface entry receptors can bind to gD and the interaction triggers fusion between the viral envelope and the cell membrane, a process that requires the participation of gB and gH-gL (reviewed in Spear and Longnecker, 2003).

Following cell fusion, the capsid-tegument structure is released into the cytoplasm of the host cell. While some of the tegument proteins remain in the cytoplasm (US11 and vhs), the viral DNA and the other tegument proteins (VP16, VP1-2) are retrogradely transported to the nuclear membrane via microtubules (Sodeik et al., 1997). At the nuclear pore membrane, the viral DNA and associated tegument proteins are released into the nucleoplasm (Roizman and Knipe, 2001).

1.7.2.3 The lytic lifecycle

During lytic infection tight regulatory and organisational alterations occur within the host cell in order to manipulate the cell for viral gene expression and protein synthesis. Lytic gene expression has been classified according to temporal expression upon cell entry into immediate early (IE or α), early (E or β) and late (L or γ) (Roizman and Knipe, 2001) gene expression.

Tegumental proteins released into the cytoplasm act to convert cellular resources from host cell protein synthesis to viral protein synthesis. Early host protein synthesis occurs in the absence of viral gene expression and is facilitated by copies of the tegumental virion host shut-off protein (vhs) released into the cytoplasm (Kwong and Frenkel, 1987). This allows an immediate shutoff after viral entry, although the gene encoding for vhs, *UL41*, is actually expressed as a late gene. Vhs is an mRNA-specific RNase which accelerates mRNA degradation of both host and viral mRNA (Kwong and Frenkel, 1987; Oroskar and Read, 1987), triggers rapid shut off of host protein synthesis, disrupts preexisting polyribosomes (Smiley et al., 2001; Smiley, 2004), and as such helps to redirect the cell from host to viral gene expression (Feng et al., 2001).

Transcription of the five IE genes, *ICP0*, *ICP4*, *ICP22*, *ICP27*, and *ICP47*, is regulated by promoters that are responsive to a viral structural protein, VP16, which is part of the tegument and transported to the host cell nucleus with the viral DNA (Roizman and Knipe, 2001). VP16 requires the presence of two host proteins, the octamer binding protein one (oct-1) and the host cell factor (HCF), to efficiently bind to cognate motifs within the IE promoter and to promote transcriptional activation of the IE genes (Mackem and Roizman, 1982; Campbell et al., 1984; Preston et al., 1988). Apart from its activation effect, VP16 has been reported to downregulate vhs expression, thus rescuing viral mRNA degradation (Mossman et al., 2000).

IE gene expression commences in the absence of viral protein expression and all IE gene products (except ICP47) are involved for the regulation of E and L gene expression. Of the IE gene products, only ICP4 and ICP27 are essential for expression of E and L genes, and thus viral replication (DeLuca et al., 1985; Sacks et al., 1985).

ICP4 activates the subsequent expression of early and late genes, whilst down-regulating its own expression and that of ICP0 (Dixon and Schaffer, 1980; DeLuca et al., 1985; Roberts et al., 1988). ICP4 can directly bind DNA (Kristie and Roizman, 1986) as homodimers (Metzler and Wilcox, 1985) and is believed to activate transcription via interaction with cellular transcription factors, such as the TATA binding protein and TFIID (Smith et al., 1993; Carrozza and DeLuca, 1996; Grondin and DeLuca, 2000).

ICP27 exerts multiple functions (Sacks et al., 1985), including the transition from early to late gene expression. By interaction with ICP4 and ICP0 IE and E gene expression is repressed, while late gene expression is activated (Sacks et al., 1985; McCarthy et al., 1989; Rice and Lam, 1994). ICP27 inhibits the nuclear localisation of ICP4 and modulates its DNA binding activity (Panagiotidis et al., 1997). ICP27 is also required for viral DNA synthesis (McCarthy et al., 1989; Uprichard and Knipe, 1996). On the post-translational level ICP27 inhibits pre-mRNA splicing and thus provides an advantage for viral mRNA that is predominantly unspliced (only four of the ca. 84 viral genes contain introns, and three of these are IE genes) (Hardy and

Sandri-Goldin, 1994; Smiley, 2004). Therefore, ICP27 collaborates with vhs for efficient shutoff of host protein synthesis and contributes to loss of host mRNAs (reviewed in Smiley, 2004). Furthermore, it has been shown that ICP27 has potential anti-apoptotic effects in human (Aubert et al., 1999; Aubert and Blaho, 1999; Zachos et al., 2001) and BHK cells (Zachos et al., 2001), thereby escaping the host cell defence mechanism to eliminate infected cells. It has been suggested that this anti-apoptotic function of ICP27, as well as ICP4, is facilitated by stabilization of bcl-2 RNA (Zachos et al., 2001).

The gene encoding for the nuclear phosphoprotein ICP0 is embedded within the short repeat regions of the HSV-1 genome, and thus is present in two copies per genome (Pereira et al., 1977; Zhu et al., 1991). ICPO is a potent activator for all three classes of viral genes IE, E and L (Cai and Schaffer, 1992; Chen and Silverstein, 1992) and is considered as a promiscuous activator for viral and non-viral genes since it can induce expression of otherwise silent genes (Nabel et al., 1988; Samaniego et al., 1998). Multiple functions have been described for ICPO including interaction with the transcription machinery (Lees-Miller et al., 1996), protein degradation (Everett et al., 1997; Everett et al., 1998; Everett, 2000), cell cyle arrest (Hobbs and DeLuca, 1999) and also the inhibition of histone deacetylation (Poon et al., 2003). Due to the fact that ICPO mRNA is anti-sense and partially complementary to LAT a potential role in latency has also been speculated (see later).

ICP22 is dispensable for virus replication and promotes late gene expression in a cell type specific manner (Sears et al., 1985). By modification of the phosphorylation state of host RNA polymerase II, ICP22 specifically represses host cell replication (Rice et al., 1995) and increases the stability and splicing of ICP0 mRNA (Carter and Roizman, 1996).

ICP47 is non-essential for virus replication in most cell types (Mavromara-Nazos et al., 1986) and inhibits the transporters of antigen processing (TAP), thereby reducing the presentation of HSV-1 epitopes on the surface of infected cells by MHC class I complexes (York et al., 1994). It has been demonstrated that the ICP47 gene product binds to TAP and prevents peptide translocation into the endoplasmic reticulum (ER)

(Hill et al., 1995). The empty MHC class I molecules remain in the ER and fail to present peptides on the surface of the infected cell (Bauer and Tampe, 2002).

With the expression of IE genes a cascade of viral gene expression is induced. Early gene products primarily encode for enzymes involved in DNA replication, followed by expression of late genes mainly encoding structural components of the virion (Honess and Watson, 1974; Honess and Roizman, 1975; Roizman and Knipe, 2001).

In the classic model of HSV-1 DNA replication, viral DNA synthesis occurs by a rolling circle producing head-to-tail concatamers of U_L and U_S that are separated by the inverted repeats (Jacob et al., 1979). During replication, the U_L and U_S segments can independently reverse their orientation by homologous recombination between the inverted repeat elements, forming four possible isomers (Jacob et al., 1979; Mocarski and Roizman, 1982; Davison and Wilkie, 1983). However, this model has recently been challenged by data suggesting that genome circularisation does not occur during lytic infection but during latency (Jackson and DeLuca, 2003).

Late gene expression requires the activities of ICP27 and ICP4 as well viral DNA synthesis (Mavromara-Nazos and Roizman, 1987). Following L gene expression, the capsid is assembled within the nucleus, the viral DNA cleaved and packaged through recognition of the packaging sequence "a" within the internal repeat of the short segment, and finally the tegument proteins associated (reviewed in Glorioso et al., 1995). The capsid-tegument complex buds through a modified patch of the nuclear membrane containing the viral glycoproteins, and thus forms the viral envelope.

Two models have been suggested for trafficking and secretion of the budded viral particles: (i) the enveloped virions fuse with the outer nuclear membrane to be released as envelope-free capsids and are re-enveloped by passaging through the Golgi compartment and subsequent release through secretory vesicles (Skepper et al., 2001; Granzow et al., 2001); (ii) the virions are released as enveloped particles from the nucleus and travel in the cytoplasm either in vesicles or in the lumen of the ER to the Golgi; after final glycoprotein maturation in the Golgi compartment they are released via a secretory pathway (Roizman and Knipe, 2001).

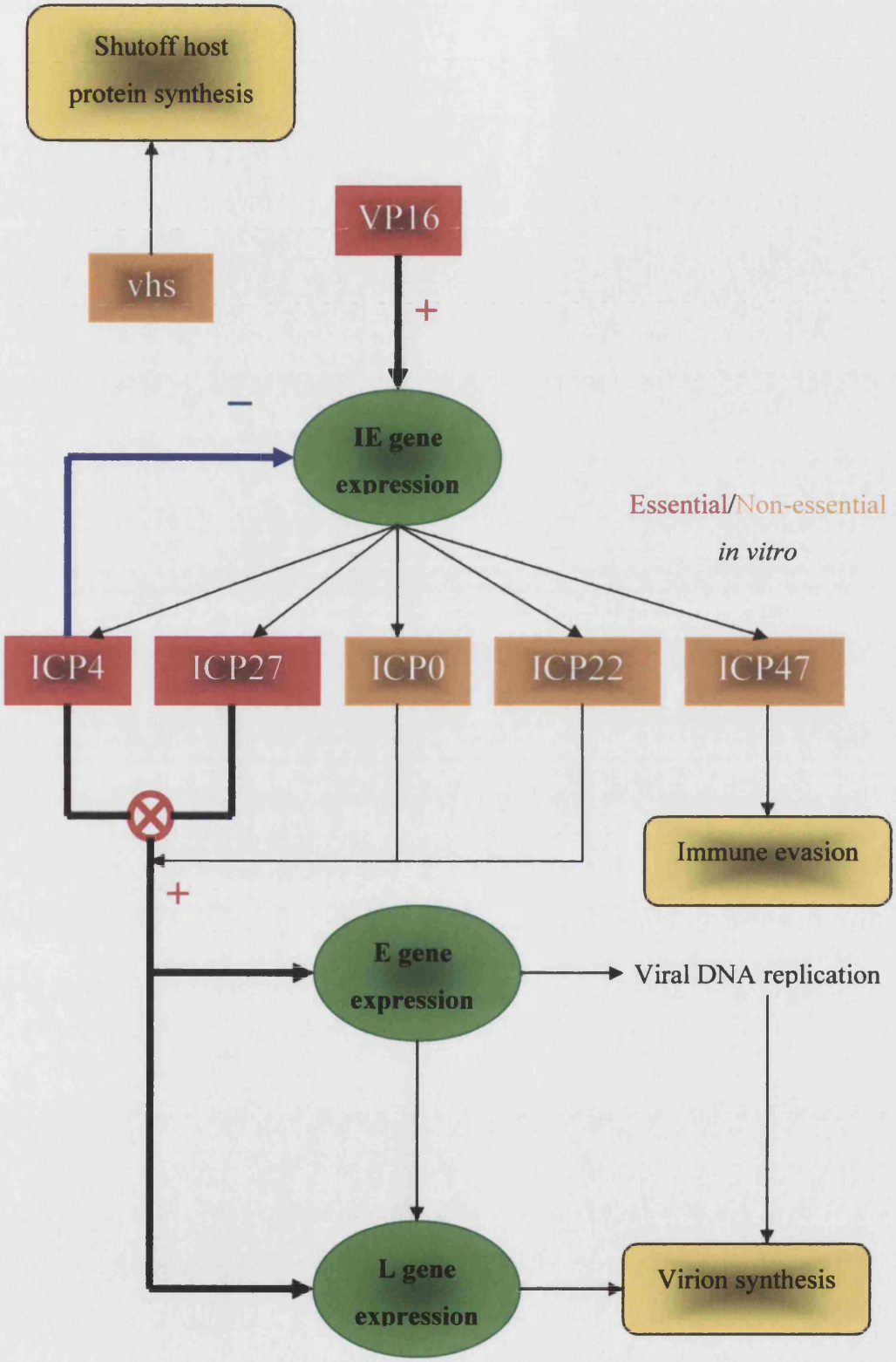


Figure 1-10: Cascade of lytic gene expression. Enhancement (+), repression (-). ICP 27 and ICP4 are both essential for induction of E and L gene expression. Adapted from Burton (2001).

1.7.2.4 The latent lifecycle

Following an initial lytic infection, HSV-1 can establish latency in sensory neurons, a state that can persist for the lifetime of the host (Mellerick and Fraser, 1987). The latency-reactivation cycle involves three major steps: establishment, maintenance, and reactivation (reviewed by Jones, 2003). However, the processes regulating the establishment or reactivation of latency are not well understood.

Establishment of latency includes viral entry followed by shutoff of viral genome expression, except of the transcription of the latency-associated transcript (LAT).

During maintenance of latency the viral genome persists as a stable episomal element and the viral genome is associated with cellular histones and adopts a chromatin-like structure (Deshmane and Fraser, 1989) that is probably not extensively methylated (Dressler et al., 1987). The LAT region is the only known transcript that is abundantly expressed during this stage of latency.

External stimuli (e.g. stress and immunosuppression) can initiate the reactivation from latency and stimulate viral gene expression followed by the lytic gene expression cascade.

The primary transcribed LAT is an 8.5 kb unstable product that is spliced into the most abundant 2 kb LAT transcript (Farrell et al., 1991; Krummenacher et al., 1997). In neurons the 2 kb LAT can also be spliced to yield 1.4 and 1.5 kb transcripts (Mador et al., 1995). The majority of LAT is not capped, lacks a consensus poly(A) addition site, and appears to be circular (Jones, 2003). Although the exact function of LAT has remained obscure, it is generally accepted that it is multifunctional, and that it plays an important but not essential role in latency (reviewed in Burton et al., 2001; Jones, 2003). LAT is predominantly nuclear localized and associated with polyribosomes, suggesting that it can be translated or regulates translation (Goldenberg et al., 1997; Ahmed and Fraser, 2001). LAT contains small open reading frames (ORFs) (Wechsler et al., 1989; Coffin et al., 1998; Dolan et al., 1998) and it has been suggested that LAT encodes for a putative protein that is potentially involved in reactivation (Thomas et al., 1999; Thomas et al., 2002). LAT is complementary to ICP0 and overlaps the ICP0 transcript, suggesting that it inhibits ICP0 expression by

antisense mechanism. Finally, it has been suggested that LAT is anti-apoptotic and enhances neuronal survival (Perng et al., 2000).

The expression of LAT is regulated by two promoters that are located upstream of the start site of LAT, latency-associated promoters 1 and 2 (LAP1 and LAP2). The promoter fragments can *cis* activate a reporter gene in transiently transfected cells (Goins et al., 1994; Chen et al., 1995). LAP1 is a TATA box containing promoter, located 28bp upstream of the primary LAT transcript, and critical for directing LAT expression in sensory neurons (Dobson et al., 1989; Mitchell et al., 1990; Deshmane et al., 1993; Chen et al., 1995). LAP2, located between LAP1 and the 5'end of the 2kb LAT, does not contain a TATA box, but includes regulatory elements frequently found in the promoters of housekeeping genes, and promotes the expression of the stable 2kb transcript under lytic conditions (Chen et al., 1995). LAP2 functions as a long-term enhancer in latently infected mice and is also required for maintaining LAP1 promoter activity (Lokensgard et al., 1994; Lokensgard et al., 1997; Berthomme et al., 2000). Within the LAT promoter region a high abundance of binding sites for cellular transcription factors can be found (reviewed in Jones, 2003) and since many of these factors can also been detected in non-neuronal cells it might explain that the LAT promoter elements can direct transgene expression in non-neuronal cells (Batchelor and O'Hare, 1992; Batchelor et al., 1994; Berthomme et al., 2000).

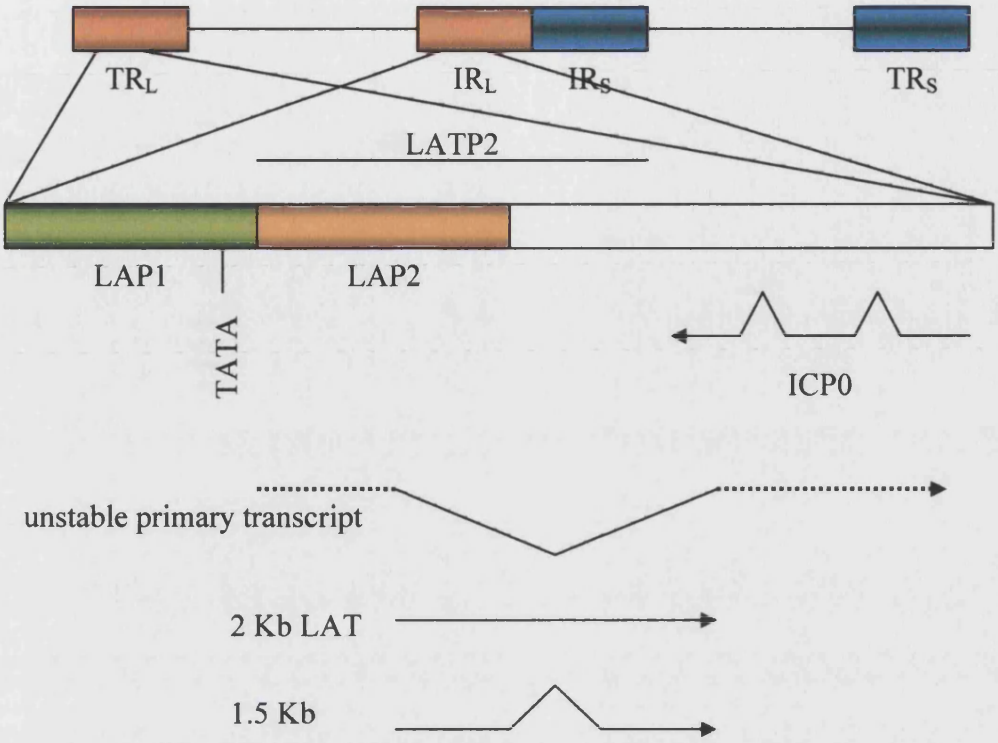


Figure 1-11: The Latency Associated Transcript (LAT).

1.7.3 HSV-1 manipulations for gene delivery

Wild-type HSV-1 infection is toxic and inevitably leads to death of the infected cells. The potential use of HSV-1 as a gene delivery vector therefore requires the elimination of pathogenicity (except applications for the destruction of e.g. neoplastic tissue) without losing the advantageous features, such as broad host range, long-term gene expression or propagation to high titres. Vector development has focused on two main strategies: construction of defective and disabled vectors.

1.7.3.1 HSV-1 amplicons (defective HSV-1 vectors)

HSV-1 amplicons, also referred to as "defective HSV vectors" (Geller and Breakefield, 1988; Geller and Freese, 1990), were originially discovered as contaminating wild-type-sized (about 150 kb) defective genomes composed of multiple repetitions of a partial HSV sequence organized in concatamers (Spaete and Frenkel, 1982). The monomers contain at least one origin of viral DNA replication and a DNA cleavage/packaging sequence. Since these virions are unable to replicate

in the absence of a helper virus, they offered a potential vector system for gene delivery. The reiterated sequence of one of these defective viruses was cloned into a plasmid vector and the generated amplicon was sufficient to direct concatameric packaging of the plasmid sequence into HSV-1 virions in the presence of a complementing helper virus (Spaete and Frenkel, 1982; Spaete and Frenkel, 1985). As multiple copies of the transgene can be inserted by this method, high level gene expression can be achieved, large sizes of foreign DNA inserted, and the construction of the amplicons is straightforward (Geller and Breakefield, 1988). However, a major drawback of this system was the use of helper virus, which physico-chemically is indistinguishable from amplicon vetors, and therefore difficult to remove (Hermens and Verhaagen, 1998). Contaminating replication-deficient helper virus can be responsible for the induction of cytopathic effects and immune responses (Johnson et al., 1992; Wood et al., 1994; Ho et al., 1995) and bears the risk of generating replication-competent virus. This has led to development of helper-virus free packaging systems by delivering the required HSV genomic DNA using cosmid (Fraefel et al., 1996) and bacterial artificial chromosome (BAC) systems (Saeki et al., 1998). By additionally deleting IE genes from these complementing vectors (Saeki et al., 2001), the generation of replication-competent virions and cytotoxicity could be significantly reduced. To date, the application of amplicons is mainly limited by the rather short-term (days to months) gene expression, that may be the result of loss of transgene DNA due to degradation, downregulation of transgene expression, or immune rejection (Sena-Esteves et al., 2000).

Successful amplicon based gene delivery has been demonstrated *in vitro* (Battleman et al., 1993; Casaccia-Bonnefil et al., 1993; Geschwind et al., 1994; Geller et al., 1995) and *in vivo* (Geller et al., 1997; Costantini et al., 1999; Sandler et al., 2002; Harvey et al., 2003; Oh et al., 2003). Geller demonstrated stable reporter gene expression in various primary neuronal cultures from the adult rat CNS using a pHSV amplicon vector driving LacZ under the immediate early 4/5 promoter (Geller and Freese, 1990). Other reports demonstrated gene delivery to cortical (Detrait et al., 2002), striatal (Freese and Geller, 1991; Aboody-Guterman et al., 1997) and mesencephalic neuronal cultures (Costantini et al., 1999), as well as embryonic stem cells (Vicario and Schimmang, 2003).

1.7.3.2 Disabled vectors

Disabled vectors have foreign genes directly inserted into the viral genome while genes involved in the production of cytopathic effects are deleted. The large size of the HSV-1 genome does not allow cloning of a restriction fragment directly into it. However, the foreign gene flanked by at least 1kb of unaltered viral DNA can be inserted by co-transfection with viral DNA (Coffin et al., 1996). As the viral DNA remains infectious, a lytic cycle proceeds and by homologous recombination, the foreign DNA is introduced into the virus at a site in the viral genome dictated by the flanking region. A small percentage of the resulting viral plaques will be recombinant virus which can be selected due to phenotypic differences (marker gene acitivity or in case of an essential gene deletion, growth in a complementing cell line).

As an approach to eliminate cytotoxicity of HSV-1 either essential or non-essential genes can be deleted. Essential genes are absolutely required for growth and pathogenicity and thus, their deletion requires complementation *in trans* for propagation in culture. Non-essential genes are only required for growth *in vivo* and therefore do not need to be complemented for virus propagation *in vitro*. Elimination of pathogenicity aims not only to prevent viral replication in cells to which transgenes are to be delivered, but also to minimise the expression of cytotoxic gene products that may still be produced even in the absence of viral replication (Burton et al., 2002).

The first generation of disabled HSV-1 vectors were deleted for non-essential genes, such as *thymidine kinase (tk)* gene (Ho and Mocarski, 1988) or the *RL1* gene (encoding for ICP34.5) (Chou et al., 1990; McGeoch et al., 1991). ICP34.5 is thought to act by inhibiting the cessation of protein synthesis associated with apoptosis in some nonpermissive, dividing cells (Chou and Roizman, 1992; Chou and Roizman, 1994; Chou et al., 1995) and thus, preventing programmed cell death in these cells to allow continued virus replication. ICP34.5 deleted viruses showed a significant decrease in neurovirulence (Chou et al., 1990) and were indeed replication-deficient in the CNS and sensory ganglia of mice (Chou et al., 1990; MacLean et al., 1991).

However, at some primary sites of infection the mutant was still found capable of replication (e.g. footpad, ependymal cells) (Robertson et al., 1992; Kesari et al., 1998), and it is believed that ICP34.5 null mutants permit viral replication in actively dividing cells but not in terminally differentiated cells (Brown et al., 1994). ICP34.5 deleted vectors expressing LacZ under control of the LAT promoter gave transient transgene expression in neuronal tissue *in vivo* (Coffin et al., 1996). However, expression of viral antigens was not abolished and caused inflammatory responses in the CNS (McMenamin et al., 1998).

Considerable effort has been undertaken to construct disabled viruses that are blocked at a very early stage in the lytic regulatory cascade to establish a state that is similar to latency (Dobson et al., 1990; Katz et al., 1990). By elimination of IE genes proceeding E and L gene expression can efficiently be aborted, and of particularly interest is the deactivation of the essential genes products ICP4 and ICP27 (Burton et al., 2002). One of the first replication-deficient HSV-1 vectors developed was deleted for ICP4 (DeLuca et al., 1985) that is unable to replicate in noncomplementing cells in culture. However, this single manipulation was not adequate to completely prevent cytotoxicity, since expression of the remaining IE gene products (ICP0, ICP22, ICP27 and ICP47) as well as ICP6 and ORF P were toxic to host cells (Johnson et al., 1994; Wu et al., 1996; Samaniego et al., 1998). As shown before (Figure 1-10) IE genes are negatively regulated by ICP4, such that infection with ICP4 disabled virus results in their overexpression causing an extensive cell death in the absence of viral replication (DeLuca et al., 1985; Krisky et al., 1998; Moriuchi et al., 2000). This has lead to development of vectors that were systematically deleted for other IE genes to reduce or eliminate the toxic effects of ICP4 deletion mutants (reviewed in Glorioso et al., 1995). However, additional deletion of ICP22 and/or ICP47 did not result in reduced cytotoxicity (Johnson et al., 1992), nor did the deletion of U_L41 (Johnson et al., 1994).

With the construction of cell lines supplying ICP4 and ICP27 *in trans* the generation and propagation of ICP4/ICP27 double mutant viruses has become possible that could also be further impaired for other non-essential IE genes (Wu et al., 1996; Samaniego et al., 1998; Krisky et al., 1998). Characterization of these vectors showed that simultaneous deletion of genes encoding for ICP4, ICP27 and ICP22 improves vector

cytotoxicity and primary neuronal cultures expressed delivered transgenes for more than 21 days *in vitro* (Krisky et al., 1998). However, in cells infected with these triple mutants, cellular DNA replication and cell division were impaired (Wu et al., 1996). Electron microscopy analysis revealed accumulation of ICP0 inclusions in the nucleus of the infected cells (Wu et al., 1996). On the other hand, viruses deleted for ICP4, ICP27 and ICP0 also showed reduced cytotoxicity but not arrest of cell division (Samaniego et al., 1997) suggesting that ICP0 is responsible for cell cycle impairment.

Quintuple IE gene deleted vectors ($ICP0^-$:$ICP4^-$:$ICP22^-$:$ICP27^-$:$ICP47^-$) have also been constructed, and are entirely non-toxic to cells (Samaniego et al., 1998). Although the vector persists for extended periods in the cells (Samaniego et al., 1998) it only grows poorly in culture and gives low levels of transgene expression in the absence of ICP0 (Samaniego et al., 1998). Therefore, this virus has little use as a vector in gene therapy. Cell lines engineered to supplement for all IE genes could improve the growth of these fully disabled vectors, but are difficult to generate due to toxic effects of the gene products (Krisky et al., 1998). It has been shown that post-translational processing of ICP0 is different in neurons than from glia (Chen et al., 2000), and it appears that ICP0 undergoes proteolytic degradation in neurons. Thus, it is hypothesised that deletion of ICP0 with the purpose of transgene delivery to neurons may not be required.

Another approach that has been undertaken to reduce vector cytotoxicity involves the inactivation of the gene encoding for VP16, the transactivator of IE promoters following virus infection (Johnson et al., 1994; Preston and Nicholl, 1997; Thomas et al., 1999; Palmer et al., 2000; Marshall et al., 2000; Lilley et al., 2001). Since IE gene expression relies on VP16 its inactivation potentially reduces the expression of the IE genes without the need to delete individually all the IE genes and construction of complementing cell lines. Since VP16 is an essential structural component of the virion, it cannot be deleted, and providing the gene product *in trans* would result in the assembly of virions containing fully functional VP16. As alternative approach, a mutation has been inserted in the VP16 gene that disrupts a domain of the protein responsible for the interaction with with Oct-1 and HCF but allows it to retain its

structural role (Ace et al., 1989). The VP16 mutation was inserted into HSV-1 strain 17+, and the resulting virus (*in* 1814) was unable to transinduce IE gene expression, expressed IE gene products at low levels and was avirulent in mice (Ace et al., 1989). Propagation of VP16 deleted viruses does not require complementation in culture and the supplementation of hexamethylbisacetamide (HMBA) allows transactivation of IE gene expression and viral growth *in vitro* (McFarlane et al., 1992). The *in* 1814 viruses have been used as backbone for further deletions and inactivations of IE genes to reduce basal expression of the encoded cytopathic proteins (Preston and Nicholl, 1997; Marshall et al., 2000). Ultimately, this has led to the development of a vector combining the VP16 mutation with deletions in ICP4, ICP27 and ICP34.5 (Lilley et al., 2001). In non-complementing cell lines this vector expressed only minimal amounts of the IE gene products ICP0, ICP22 and ICP47. It can easily been propagated to high titre stocks, allows high gene delivery to neuronal cells *in vitro* and *in vivo*, and maintains expression for extended periods depending on the chosen promoter (Lilley et al., 2001).

1.7.3.3 Using LAT elements for transgene expression

The fact that HSV-1 can establish a latent state during which no lytic genes are expressed, but a region of its genome remains transcriptionaly active (LAT), makes it very attractive for development of vectors exploiting the LAT region for potential long-term transgene expression. Since the LAT genes are not essential for establishment, maintenance, or reactivation from latency (see above), it is possible to insert transgenes within the LAT loci, disrupting the LAT genes and using the LAT *cis*-acting regulatory sequences to drive transgene expression (Burton et al., 2001).

Several studies have shown that the LAP promoter region can be used for long-term transgene expression in the peripheral nervous system (PNS) (Goins et al., 1994; Lachmann and Efstathiou, 1997; Goins et al., 1999; Palmer et al., 2000). In summary, these studies have shown that the LAP1 seems to drive high-level short-term reporter gene expression when placed within the LAT region or ectopically from another region in the genome. LAP2, on the other hand, allows extended transgene expression, but only at low levels. Combining the high-level expression features of

LAP1 with the long-term activity associated with LAP2 by placing a *lacZ* gene downstream of an internal ribosome entry site (IRES), did indeed allow increased and long-term transgene expression (Lachmann and Efstathiou, 1997; Marshall et al., 2000). Transgene expression could further be improved with the use of heterologous promoters by inserting CMV or MMLV promoters adjacent to the LAP1 and LAT P2 fragment (LAP2 plus 700bp at the 3'end) (Palmer et al., 2000).

While HSV-1 naturally establishes latency in sensory neurons, its activity within neurons of the CNS is less well understood. Following an acute infection, replication-competent and neuro-attenuated vectors persist in CNS neurons where they transcribe the LAT region (Drummond et al., 1994; Kesari et al., 1996; Smith et al., 2000) Although long-term expression using the latency promoter system has been demonstrated with a replication-competent attenuated vector (Smith et al., 2000), only transient gene expression was observed with replication-deficient viruses (Oligino et al., 1998). Intrastriatal injections of a vector deleted for ICP0 and ICP4 that additionally contained an insertion in the VP16 gene abolishing its transactivating activity, showed stable reporter gene expression from LAP for up to 4 weeks (Scarpini et al., 2001). Cells expressing the reporter gene were found around the injection site and in distal regions to it, such as cortex, thalamus, and substantia nigra. However, transgene expression dramatically dropped after 7 weeks post injection, which could not be explained with the death of the infected cells since viral genomes were detected for up to 24 weeks in the CNS (Scarpini et al., 2001). Strong reporter gene expression was also detected in a highly disabled vector ($ICP27^-$:$ICP4^-$:$ICP34.5^-$:VP16mutated) driving the *lacZ* gene under a LAP1/LATP2/CMV heterologous promoter (Lilley et al., 2001). After intrastriatal injection the vector undergoes retrograde transport to cell bodies located in the substantia nigra and exerts significant transgene expression for up to 1 month *in vivo*. In conclusion, the studies have shown that using the latency promoters extended transgene expression can be achieved not only in the PNS but also in neuronal tissue of the CNS.

Viral promoters other than the latency promoters give only short-term transgene expression in CNS neurons (Bloom et al., 1995; Howard et al., 1998; Yamada et al., 1999; Marconi et al., 1999).

1.8 Gene therapeutic approaches for PD

Gene therapy can be defined as the delivery of engineered genetic material into a host cell such that it can perpetuate the production of the desired gene product. In the case of PD most gene therapeutic approaches have focused on either the delivery of enzymes involved in biosynthesis of dopamine or the expression of neurotrophic factors. Other gene products considered for use have roles in the protection against apoptosis or exhibit anti-oxidative properties. The first gene therapeutic trial for PD has been launched in August 2003 using a combination of gene delivery approach and surgical treatment. Preliminary results are expected at the end of 2004 (see below). Depending on the route of administration gene therapy for PD can be divided into *ex vivo* gene therapy or *in vivo* gene therapy. In *ex vivo* approaches the patients cells are manipulated *in vitro* prior to being transplanted into the host, while *in vivo* approaches involve direct gene transfer into the host somatic cell *in situ* (Le and Frim, 2002). The fact that the depletion of a single, well defined group of neurons causes PD, along with the availability of well characterized animal models, makes PD potentially amenable to gene therapy.

1.8.1 *Ex vivo* gene therapy strategies

The advantage of *ex vivo* approaches is that gene expression can be assessed and manipulated prior to implantation. Genetically manipulated cells can be considered as biological "minipumps" releasing therapeutic factors at the grafting site. However, a frequent occurrence and significant limitation is the low level of cell viability that is found after grafting.

Attempts have been described using immortalised cell lines expressing L-DOPA and TH (Freed et al., 1986; Horellou et al., 1990; Date et al., 2000), encapsulated tumor cell lines (Lindner et al., 1995) or GDNF expressing neural stem cell lines (Akerud et al., 2001). However, major concerns regarding cell lines arise due to the possibilities of oncogenic spread, and the use of tumor cell lines in human clinical gene therapy seems unlikely.

Other cell sources with potential therapeutic value after genetic modification are primary fibroblasts, astrocytes and neural precursor cells. Fibroblasts have been engineered to express neurotrophic factors such as BDNF (Frim et al., 1994; Levivier et al., 1995) and GDNF (Perez-Navarro et al., 1996), as well as enzymes involved in dopamine synthesis (Wolff et al., 1989). The advantage of using fibroblasts is that they can easily be harvested from the patient, e.g. by skin biopsy, and transplanted autologously after modification *in vitro*, thereby reducing the risk of graft rejection.

In similar approaches astrocytes have successfully been modified and transplanted (Lundberg et al., 1996; Ridet et al., 1999). Astrocytes play an important role as supportive neural cell type and their long lifespan and efficient secretory system make them attractive candidates for gene delivery (Lin et al., 1997; Ridet et al., 1999; Serguera et al., 2001). Gene expression can be targeted by using the promoter for glial fibrillary acidic protein (GFAP) as it has for instance been shown for the long term expression of TH in a rat model of PD (Cortez et al., 2000). Other examples using astrocytes as cell vehicle have demonstrated expression of neurotrophic factors, such as GDNF (Ericson et al., 2002) or BDNF (Yoshimoto et al., 1995). However, it has been suggested that astrocyte maturation *in vitro* might be accompanied by biochemical and functional changes that limit their capacity to support axon growth (Ostenfeld et al., 2002).

Neural precursor cells have also been engineered to express therapeutic transgenes. For instance, neurospheres derived from rat embryonic striatal tissue have been transduced with GDNF expressing lentivirus and were grafted into hemiparkinsonian rats where GDNF increased the survival and neurite outgrowth of precursor cells, however, an approach that did not improve behavioural impairment (Ostenfeld et al., 2002). An advantage of using precursor cells is that they retain their migratory potential and hence, spread through a larger region and often move towards the area of damage (Hoehn et al., 2002). Furthermore, it might become possible to generate precursor cells from the patients themselves to be genetically manipulated, thereby reducing the risk of immune rejection. As discussed elsewhere in this study, neural precursor cells could ultimatively been engineered to predifferentiate into an immature dopamine phenotype and fully mature post implantation. In this model

precursor cells would terminally differentiate and integrate as dopaminergic neurons *in situ*, thereby increasing survival and functional implementation into the existing circuitry. However, the factors required to induce an immature dopamine phenotype still need to be elucidated and it is not known if in the adult, damaged brain the clues are present that are required for a terminal differentiation into a dopaminergic neuronal phenotype.

1.8.2 *In vivo* gene therapy strategies

For a direct gene transfer of genetic to the host somatic cell, viral vectors have become the prefered tool due to higher gene delivery efficiency compared to non-viral system. Although many viral vectors are in common use, the optimal viral vectors needs to be assessed for each specific application. Vector choice is determined by efficiency and specificity for the the target cell, the duration of required transgene expression, whether regulation of the transgene expression is required, and the level of toxicity that is tolerated. Similar to *ex vivo* approaches, current *in vivo* strategies focus on prevention of cell death and delivery of genes involved in dopamine biosynthesis.

Local expression of apoptotic inhibitors prevents the loss of toxic induced dopaminergic cell loss as shown by HSV-1 delivered bcl-2 (Yamada et al., 1999), adenovirus delivered human neuronal apoptosis inhibitor protein (NAIP) (Crocker et al., 2001) or AAV delivered apoptotic protease-activating factor-1 (apaf-1) (Mochizuki et al., 2001). Although apopotosis has been reported as one possible mechanism of cell death in the SN of PD (reviewed by Vila and Przedborski, 2003) its role is still controversial. Furthermore, prolonged expression of anti-apoptotic factors has not been fully explored and as some of these factors are proto-oncogenes, their therapeutic use provides safety concerns (Burton et al., 2003).

The most promising neurotrophic factor to prevent neurodegeneration and restore functionality is GDNF (see before) and its therapeutic value has been demonstrated by direct delivery to rodents and primates using lentiviruses, adenoviruses, adeno-associated virus (for review on these vectors see Bjorklund et al., 2000) and HSV-1

(Natsume et al., 2001). The studies showed robust GDNF expression after direct gene transfer into the striatum or substantia nigra, trophic effects in various animal models of PD, and restorative effects even after the toxic insult has occurred. However, long-term expression of lentivirus delivered GDNF induced aberrant sprouting accompanied by spontaneous abnormal motor behaviour (Georgievska et al., 2002) raising safety concerns over the long-term effects of GDNF. In other studies combined expression of GDNF with anti-apoptotic factors, such as X-linked inhibitor of apoptosis (XIAP) (Eberhardt et al., 2000), or bcl-2 (Natsume et al., 2001) showed synergistic effects on cell survival and functional recovery.

Early gene therapy studies for PD focused on the delivery of enzymes involved in the metabolism of dopamine, such as tyrosine hydroxylase, aromatic acid decarboxylase (AADC) or GTP-cyclohydrolase I (GCH1) that synthesises a cofactor required for AADC. Multiple vector systems have been constructed to express either of these factors or combinations (Burton et al., 2003), with the best result obtained from simultaneous expression of all three enzymes. Although enhanced dopamine production can be achieved accompanied by behavioural improvement, it is unclear whether the non-physiological sustained delivery of dopamine will alleviate the problems of adverse effects, similar to those experienced with the use of levopoda.

Surprisingly, the first clinical phase I trial for PD that has been approved by the US Food and Drug Administration that will try to reduce the overstimulation of the globus pallidus interna through production of the inhibitory neurotransmitter gamma–amino butyric acid (GABA) using AAV (During et al., 2001). AAV expressing glutamic acid decarboxylase (GAD), a key enzyme in the synthesis of GABA, will be injected into the STN. Since the STN is disinhibited in PD leading to symptoms such as tremor, rigidity and bradykinesia, GABA expression is expected to re-inhibit the STN and thus, to ameliorate the symptoms. Virus injection will be performed together with unilateral STN stimulator implantation. In case that GAD expression has an unanticipated deleterious effect, then the STN can be either electrically silenced or ablated using the stimulator without additional surgery being required. Significant setbacks have been experienced in recent gene therapy clinical trials due to the death of a patient treated for a partial deficiency of the liver enzyme ornithine

transcarbamylase (Lehrman, 1999) using adenoviral vectors and two children developing vector induced leukaemia after treatment for severe combined immunodeficiencies (SCID) (Hacein-Bey-Abina et al., 2003) with disabled retroviruses. Therefore, it seems that safety issues will play a particularly important role in the described trial for PD. Viruses are injected into a region of the brain that is sometimes surgically removed as standard treatment and thus offers a fallback position should the safety of the gene transfer become an issue (Howard, 2003). However, by using AAV the risk of viral integration into proto-oncogenes and other host genes still remains, although at a very low level considering the limited degree of AAV integration and that a non-dividing cell population is transduced.

1.9 Thesis aims

The previous sections have shown that the cause of Parkinson's disease is still unknown, and so far all available therapies aim to treat the major symptoms of the disease rather than the cause. Since the most prominent characteristic of the disease is the almost complete loss of midbrain dopaminergic neurons, a main focus has been the replacement of these cells. Various cell sources have been suggested, but the diverse developmental potential and self-renewal capacity make stem cells particularly interesting.

Foetal and adult neural precursor cells have become a promising cell source for the generation of dopaminergic neurons since they generally do not involve ethical concerns or form teratoma, the main disadvantages of embryonic stem cells. However, signals required for the conversion of multipotent precursor cells into dopaminergic neurons have remained obscure. Several viral vector systems have been described to deliver genetic material to neural precursor cells. To date no thorough studies have been published describing the use of replication-deficient HSV-1 as a vector system for neural precursor cells.

The work presented in this thesis aims to evaluate the potential of replication-disabled HSV-1 as a gene delivery vector for neural precursor cells. The main focus will be the study of gene delivery efficiency and cellular integrity of the transduced precursor cell. Optimised vectors will be modified to deliver instructive genes (*ShhN*, *FGF2*, *FGF8*, *Nurr1*). These factors have been chosen since they are known to be required for the embryonic development of dopaminergic neurons, as well as playing an important role in the generation of dopamine neurons from embryonic stem cells. The effects of the virally delivered transgenes on neural precursor cells will be studied with respect to dopamine genesis aimed at providing techniques for converting neural precursors into fully functional dopaminergic neurons either prior to transplantation/implantation or directly *in vivo*.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Materials

Unless otherwise stated, all chemicals were purchased from Merck Ltd. (Poole, Dorset, UK), Boehringer Mannheim (Lewes, East Sussex, UK) or Sigma Chemical Company Ltd. (Poole, Dorset, UK) and were of analytical grade.

General disposable plasticware was purchased from Sterilin (Stone, Staffordshire, UK) or Greiner (Stonehouse, Gloucester, UK).

Additional laboratory materials and reagents were supplied from one of the following: Insight Biotechnology Ltd. (London, UK); Nunc (Roskilde, Denmark); Amersham International plc. (Little Chalfont, UK); Qiagen (Chatsworth, USA); Difco Laboratories (Detroit, USA); Gibco-BRL Life Technologies Ltd. (Paisley, Renfrewshire, UK); Bio-Rad (Hemel Hempstead, UK); Promega Corporation (Madison, Wisconsin, USA); Whatman International Ltd. (Maidstone, Kent, UK); Pharmacia Biotechnology Ltd. (St Albans, UK); Millipore Ltd. (Watford, UK); Stratagene Ltd. (Cambridge, UK); Marligen Bioscience Inc. (USA); New England Biolabs Inc. (Hitchin, Hertfordshire, UK), Molecular Probes (Leiden, Netherlands), Chemicon (Temecula, USA), TCS Cell Works (Buckinghamshire, UK), DAKO (glostrup, Denmark).

All oligonucleotide primers were constructed by Genosys (Pampisford, Cambs, UK).

2.1.1 Standard buffers and solutions

PBS: NaCl (137mM), KCl (2.7mM), $Na_2HPO_4.7H_2O$ (4.3mM), KH_2PO_4 (1.4mM)

PB: Na_2HPO_4 (72mM), NaH_2PO_4 (28mM), pH 7.2

TE: Tris-HCl (10mM), EDTA (1mM), pH8.0

TAE: Tris base (400mM), sodium acetate (200mM), EDTA (20mM), pH8.3

TBE: Tris base (89mM), boric acid (89mM), EDTA (2mM), pH8.0

Luria Bertani (LB) media: 1% (w/v) Bacto®-tryptone
1% (w/v) NaCl

0.5% Bacto®-yeast extract

2.1.2 Bacterial strains

The XL1-Blue (XL1-B) (Stratagene Ltd.) strain of *Escherichia coli* of genotype recA1 endA1 gyrA96 thi-1 hsdR17 supE44 rel A1 lac [F'proAB $Lacl^q$ ZΔM15, TN 19 $(Tet)^r$] was used for all plasmid clonings.

2.1.3 Materials for Molecular Biology

Restriction and modifying enzymes and buffers (Promega, Madison, Wisconsin, USA)
Bacto®-agar, Bacto®-tryptone, yeast extract (Difco Laborotories, Basingstoke, UK)
Ammonium persulfate, TEMED (Bio-Rad, Hemel Hempstead, UK)
Concert™ "midi-prep" plasmid extraction kit, 1Kb DNA ladder (Gibco-BRL-Lifetechnologies Ltd., Renfrewshire, Scotland, UK)
Gel band extraction kit, Rainbow™ couloured protein full range molecular weight markers, Hybond-C and Hybond-N membranes (Amersham International plc., Little Chalfont, UK)
Thermocyler (Eppendorf, Hamburg, Germany)

2.1.4 Recombinant proteins

All recombinant proteins were purchased from R&D and reconstituted in PBS containing 0.1% bovine albumin serum and stored in aliquots at -80°C. Mouse recombinant fibroblast growth factor 8b (FGF8b, #423-F8) was prepared at a stock concentration of 50μg/ml; human recombinant basic fibroblast growth factor (FGF2, #233-FB) at a stock concentration of 20μg/ml; mouse recombinant sonic hedgehog N-terminal (ShhN, #461-SH-025) at a stock concentration of 50μg/ml.

Heparin (Sigma, H3149) was prepared in DMEM/F12 media at a stock concentration of 5mg/ml and stored in aliquots at -80°C.

2.1.5 Materials for surgery and dissection

Halothane (Rhône Mérieux Ltd., Harlow, Essex, UK)
Dissecting microscope (Olympus SZ40)
Hamilton microliter syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland)
Micro1 syringe pump controller Ultra micropump 1 (World Precision Instruments, USA)
Ultra micropump 1 (World Precision Instruments, USA)
Foredom Micro Motor MH1 45 (MHC, USA)
Surgical instruments (Fine Science Tools, Heidelberg, Germany)
Expiral (Sanofi Animal Health, Watford, UK)
Paraformaldehyde (Sigma, UK)
Michelle clips (Biological Services, UCL, UK)
Tissue chopper (McIlwan, USA)

2.1.6 Materials for tissue preparation

Cryostat, Leica CM1900 (Leica Instruments, Nusloch, Germany)
Microscope slides and coverslips (BDH Labaratory supplies, Poole, Dorset, UK)
Antifade mounting media (Molecular Probes, Leiden, Netherlands)

2.1.7 Materials for neural progenitor culture

Dulbecco's modified Eagle medium (DMEM) (11965-118; Gibco-BRL, USA)
Neurobasal® medium (21103-049, Invitrogen, UK)
Ham's F-12 (1x solution) without L-glutamine (12422; ICN, Ohio, USA)
Accutase™ (TCS Cell works, Buckinghamshire, UK)
Poly-L-lysine (PLL) (P5899; Sigma, UK)
Poly (2-hydroxyethyl methacrylate) (Poly-HEMA) (P-3932; Sigma, UK)
0.1% Trypsin (T4424; Sigma, UK)
Trypsin inhibitor (T6522; Sigma, UK)
DNAse (D4527; Sigma, UK)
Laminin (L2020; Sigma, UK)

B27 supplement (15420-062; Gibco-BRL, UK)
Human basic fibroblast growth factor 2 (FGF2) (233-FB; R&D Systems, UK)
Heparin (H3149; Sigma, UK)
Bellco German glass round coverslips (Bellco, Germany)

2.1.8 Materials for Immunolabelling

Rabbit Anti-Tyrosine Hydroxylase polyclonal (P40101-0; Pel-Freez, Arkansas, USA)
Rabbit Anti-Glial Fibrillary Acidic Protein (GFAP) polyclonal IgG (Z0334; Dako, Glostrup, Denmark)
Mouse Anti-Neuronal Nuclei (NeuN) monoclonal IgG1 (MAB377; Chemicon, USA)
Rabbit Anti-Nurr1/Not1 polyclonal (AB5778; Chemicon, USA)
Mouse Anti-β-Tubulin Isotype III (TuJ1) monoclonal IgG2b (T8660; Sigma, UK)
Rabbit Anti-FGF-2 polyclonal (sc-79; Santa Cruz Biotechnology, USA)
Mouse Anti-Oligodendrocyte marker O4 monoclonal IgM (MAB345; Chemicon, USA)
Mouse Anti-galactocerebroside GalC monoclonal IgG3 (MAB342; Chemicon, USA)
Alexa Fluor® 350 goat anti-mouse IgM (A31552; Molecular Probes, Netherlands)
Alexa Fluor® 546 goat anti-rabbit IgG (A11010; Molecular Probes, Netherlands)
Alexa Fluor® 488 goat anti-rat IgG (A11006; Molecular Probes, Netherlands)
Alexa Fluor® 546 goat anti-mouse IgG1 (A21123; Molecular Probes, Netherlands)
Alexa Fluor® 488 goat anti-mouse IgG2b (A21141; Molecular Probes, Netherlands)
Goat Anti-Rabbit IgM+IgG rhodamine conjugated (4010-03; Southern Biotechnology, Birmingham, USA)
Goat Anti-Mouse IgG rhodamine conjugated (1031-03; Southern Biotechnology, Birmingham, USA)
Goat Anti-Mouse IgG fluorescine conjugated (1031-02; Southern Biotechnology, Birmingham, USA)
Mouse Anti-Nestin monoclonal IgG1 (MAB353; Chemicon, USA)
Rat Anti-BrdU monoclonal IgG2a (ab6326; abcam, Camebridge, UK)
Goat Anti-FGF8 (N18) polyclonal (sc-6958; Santa Cruz Biotechnolgy, USA)
Goat Anti-ShhN (N19) polyclonal (sc-1194; Santa Cruz Biotechnolgy, USA)
Mouse Anti-GAPDH IgG1 (#4200, Ambion, UK)

Anti-goat IgG horseradish peroxidase (P0160, Dako, High Wycombe, Bucks, UK)
Anti-rabbit IgG horseradish peroxidase (P0217, Dako, High Wycombe, Bucks, UK)
Anti-mouse IgG horseradish peroxidase (P0260, Dako, High Wycombe, Bucks, UK)
Antifade mounting media (P7481, Molecular Probes, Leiden, Netherlands)
4',6-Diamidino-2-phenyindole (DAPI) (D9564, Sigma, UK)
Hoechst 33258 (B1155, Sigma, UK)

2.2 Molecular Biology methods

2.2.1 Bacterial growth conditions

XL1-Blue cells were grown in sterile Luria Bertani (LB) (1% w/v Bacto®-tryptone, 1% w/v NaCl, 0.5% w/v Bacto®-yeast extract) media (autoclaved at 120°C for 20 mins at 10 lb square $inch^{-1}$) containing either no antibiotic or 50 μg/ml of tetracycline overnight (O/N) in a Gallenkamp™ orbital shaker at 200 rpm. Stocks of tetracycline were made at 5 mg/ml in ethanol and stored at -20°C. Colonies of XL1-B were isolated by growing on LB plates containing 2% Bacto®-agar and 12.5 μg/ml of tetracycline.

2.2.2 Transformation of bacteria

Competent *E. coli* cells were prepared using a standard calcium chloride technique (Sambrook et al., 1989). Single bacterial colonies were selected and grown O/N in 10 ml of LB containing no antibiotic. 100 μl of this starter culture was used to inoculate 100 ml of LB containing no antibiotic up to an OD_{580} of approximately 0.5 units. The bacteria were then pelleted by centrifugation at 3500 rpm for 10 mins at 4°C and any excess LB was discarded. The cells were resuspended in 10 ml of ice-cold 100 mM $CaCl_2$ and incubated on ice for 30min. The cells were then pelleted by centrifugation as before and resuspended in 4 ml of ice-cold $CaCl_2$. The cells were then left on ice until required and used within 48 hours.

200 µl of competent cells were transformed upon addition of DNA and subsequent incubation on ice for 30 min. The cells were then heat shocked for 90 seconds at 42°C and returned to ice for a further 2 min. 800 µl of LB was added to the cells and the suspension was incubated in the orbital shaker for 1 hour at 37°C/200 rpm. The cells were pelleted (10 min/3500 rpm), resuspended in 100 µl of LB and subsequently plated onto LB agar plates containing the appropriate antibiotic selection, either 50 µg/ml of ampicillin. If detection of β-galactosidase was required, the plates used contained 50µl of a 20mg/ml stock of 4-chloro, 5-bromo, 3-indolyl-β-galactosidase (X-Gal) dissolved in dimethyl formamide.

2.2.3 Small scale plasmid DNA extraction (mini-prep)

The 'mini-prep' DNA extraction method used is based on an alkaline lysis method previously described (Birnboim and Doly, 1979). Single colonies of transformed cells were used to inoculate 3 ml of LB containing the appropriate antibiotic selection, and were incubated O/N in an orbital shaker (37°C/200 rpm). The cells from 1.5 ml of O/N culture were pelleted by centrifugation in a bench top microcentrifuge at 13000 rpm for 2 min and resuspended in 100 µl of resuspension buffer (50 mM Tris-HCL pH 7.5, 10 mM EDTA pH8). Bacteria were then lysed by addition of 200 µl of lysis buffer (200 mM NaOH, 1% (v/v) Triton X-100) and neutralised by the addition of 150 µl of neutralisation buffer (3 M sodium acetate pH5.5). The cell lysate was then centrifuged for 3 min at 13000 rpm and the pelleted precipitate was removed and discarded. 500 µl of isopropanol was then added to the supernatant, which was vortexed and centrifuged for 20 min at 13000 rpm to pellet the DNA. The supernatant was discarded and the DNA was washed with 70% ethanol, pelleted (5min at 13000rpm), dried under vacuum and resuspended in 50 µl of double-distilled water (ddH_2O) containing 20 µg/ml RNase A. Plasmid DNA was stored at –20°C.

2.2.4 Large scale plasmid DNA preparation (midi-prep)

Single colonies from a bacterial plate or approximately 100 µl of a suspension bacterial culture were used to inoculate 400 ml of LB containing the appropriate

antibiotic selection. The inoculated medium was incubated O/N in an orbital shaker at 37°C/200 rpm. 100 ml of the O/N culture were spun down at 3000 rpm for 10 min. Plasmid DNA was then extracted using the Midi-Prep Kit (Gibco-BRL) following the manufacturer's instructions. A typical yield of DNA using this method was 100 μg, which was resuspended in 100 μl of ddH_2O.

2.2.5 Viral DNA preparation

A well of a 6 well plate of virally infected cells at complete cytopathic effect (CPE) was harvested by adding 1ml of DNAzol (Helena Biosciences, Sunderland, UK). The DNA was precipitated by adding 0.5 volumes of absolute ethanol and washed twice in 75% ethanol. Ethanol was removed and the DNA pellet dried for 5min at RT. The DNA was resuspended in 200μl of 8mM NaOH and incubated O/N at 4°C on a rotating wheel. For neutralization 23μl of 0.1M HEPES were added and the DNA stored at -20°C.

2.2.6 Restriction enzyme digestion

Plasmid DNA was enzymatically digested for analysis or isolation of DNA fragments. Analytical digests were performed in a total volume of 20μl, containing 5μl of mini-prep DNA or 1μl of midi-prep DNA. 10 units of each enzyme were added and the appropriate buffer used at 1x concentration. The volume was made up to a final of 20μl with ddH_2O. Digests were incubated for 1 hour at the appropriate temperature. Depending on the size of the fragments of interest the digested DNA was run on a 0.5-1.5% agarose gel and the bands visualized on a UV transilluminator.

Restriction digests required for isolation of DNA fragments were carried out in a total volume of 100 μl containing approximately 5 μg of midi-prep DNA, 10units of each enzyme and the recommended buffer at 1x concentration. Digests were incubated O/N at the appropriate temperature and then run on a 0.5-1.5% agarose gel. DNA bands were visualised on a UV transilluminator and the bands of interest were carefully excised using a scalpel. As required DNA was extracted from the agarose using the

GFX™ PCR and Gel Band Purification Kit (Amersham) according to manufacturer's instructions. The DNA was then eluted in a final volume of 15 µl.

2.2.7 Agarose gel electrophoresis

0.5-1.5% (w/v) agarose gels were made using 1x TAE (0.4 M Tris base, 0.2 M sodium acetate, 20 mM EDTA pH8.3). Ethidium bromide was added to a final concentration of 0.5 µg/ml. Approximately 0.1 volume of 10x loading buffer (1x TAE, 50% v/v glycerol, 0.025% bromophenol blue) was added to DNA samples prior to loading. 1 kilobase (kb) ladder DNA marker was used size marker. DNA was electrophoresed at 80-120mA mA until the fragments were well separated. Bands were visualised on a UV transilluminator and photographed with Polaroid film.

2.2.8 Blunt-end reactions

For blunt ending, overhangs were treated with T4 DNA polymerase. After restriction digest, 1 µl of a 25 mM stock of dNTPs (dATP, dCTP, dTTP, dGTP) and 15 units of T4 DNA polymerase were added directly to the reaction. The reaction was incubated for 30 min at 37°C. T4 DNA polymerase was heat inactivated at 80°C for 20 min and then cooled on ice prior to the addition of further enzymes.

2.2.9 Phosphatase treatment

To prevent possible religation of vector ends, the vector DNA was dephosphorylated using calf intestinal alkaline phosphatase (CIAP). Vector DNA was extracted using the GFX™ PCR and Gel Band Purification Kit (Amersham) according to manufacturer's instructions. DNA was resuspended in a total volume of 50µl ddH_2O containing 1x phosphatase buffer and 5 units CIAP. The reaction was incubated for 45min at 37°C, DNA again extracted as before and resuspended in 10µl ddH_2O.

2.2.10 DNA ligations

Ligations were performed in a total volume of 30μl. Purified fragments of vector and insert were usually used in a ration of 1:3 (vector:insert) measured according to its approximate amount from a small sample run on an agarose gel. The liagtion mix was made up with 1x ligase buffer and 3units of T4 DNA ligase in ddH_2O. The ligation mix was incubated for 1.5 hours at RT (cohesive ligation) or O/N at 16°C (blunt ligation). The ligation reaction was transformed into XL1-1B cells (2.2.2).

2.2.11 DNA sequencing

For sequencing of PCR amplified inserts cloned into the pGemT easy vector (Promega) T7 and SP6 primers were used. Plasmid DNA was prepared at a concentration of 100ng/ml in ddH_2O and the sequencing performed at external facilities (DNA Sequencing Facility of the Biochemistry Department at the University of Camebridge, UK).

For sequencing of the Nurr1 gene internal primers were required. The following primer sequences were used:

Primer 1: 5' CCAGATGCGCTTTGACGG
Primer 2: 5' CCGTCAAAGCGCATCTGG
Primer 3: 5' CCACGCACGCATTGCAAC
Primer 4: 5' GTTGCAATGCGTGCGTGG

2.2.12 RNA extraction

All plastic tissue ware and equipment was pretreated with RNA ZAP (Sigma) or soaked in 0.1N NaOH. Tissue was dissected and immediately frozen in liquid nitrogen and stored at -80°C until RNA was prepared. Neurospheres were settled down, medium removed and immediately resuspended in TRIzol (Invitrogen). Resuspended neurospheres were stored at -80°C as required.

Tissue or neurospheres were resuspended and homogenated in 1ml TRIzol (Invitrogen) by pipetting. Homogenate was incubated for 5min at RT, 200µl chloroform added and mixed by inverting for 15sec. Samples were incubated for 3min at RT, centrifuged for 15min at 12,000 rpm at 4°C and the upper aqueous phase transferred into a new tube. RNA was precipitated by adding 500µl isopropanol, incubation for 10min at RT and subsequent centrifugation for 10min at 12,000 rpm at 4°C. The RNA pellet was washed with 75% EtOH, centrifuged for 5min at 7500g at 4°C, the ethanol removed and the pellet air dried for 10min at RT. The RNA was resuspended in 30µl DEPEC ddH_2O and incubated for 10min at 55-60°C. The RNA concentration and quality was assessed by measuring the A260/A280 ratio which was expected to be between 1.7 and 2.0. The RNA concentration was calculated based on the assumption that 1 OD_{260} equals 40µg/ml RNA. RNA was stored in aliquots at -80°C.

2.2.13 DNAse treatment

For RT-PCR reaction the extracted RNA was DNAse treated in order to remove genomic DNA contaminations. 5µg RNA were prepared with DEPEC ddH_2O in a total volume of 20µl DNAseI mix containing 1x reaction buffer and 2µl amplification grade DNaseI (Invitrogen). The DNAse mix was incubated at RT for 15min and DNAse treatment stopped by adding 1µl of 25mM EDTA and incubation for 15min at 65°C. The mixture was cooled down, collected by brief centrifugation and was directly used for reverse transcription.

2.2.14 First strand cDNA synthesis

Neurosphere RNA was reverse transcribed and subsequently amplified in two separate reactions. Tissue RNA was reverse transcribed and amplified with gene specific primers using One-Step RT-PCR kit (Qiagen).

First strand cDNA synthesis of 1µg neurosphere RNA was performed using the SuperScript First Strand Synthesis System™ (Invitrogen). The RT-PCR reaction was set up and carried out according to manufacturer's instructions.

2.2.15 PCR amplification

PCR amplifications for semi-quantitative RNA analysis were performed using Platinum Taq DNA Polymerase (Invitrogen). PCR reactions were set up using 2μl of prepared cDNA (2.2.14) in a total volume of 50μl according to manufacturer's instructions. The PCR mix was gently mixed and transferred to the thermocycler. The standard PCR program used included a hot start at 94°C for 2min, denaturation at 94 for 15 sec, annealing at the indicated temperature for 30sec and extension at 72°C for 1 min. The number of PCR cycles was chosen as indicated. A final extension step at 72°C for 10min was performed. The PCR products were analysed on 1-2% agarose gels.

2.3 Cell culture methods

All tissue culture preparations were carried out under sterile conditions in a laminar flow safety cabinet. All viral preparations were performed under Health and Safety Category 2 Conditions.

All cell lines and viruses were stored long term in liquid nitrogen and during culture were maintained at 37°C in a 5% CO_2 incubator in a humidified atmosphere.

2.3.1 Baby Hamster Kidney Cells

Baby Hamster Kidney cells (BHKs) Clone 13 (Macpherson and Stoker, 1962) were provided by Imperial Cancer Research Fund (ICRF), London, UK. BHK cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 100 units/ml penicillin/streptomycin (also refered to as serum free medium, SFM) supplemented with 10% Fetal Calf Serum (FCS) and is referred to as full growth media (FGM). BHK cells were passaged by washing in Hank's Balanced Salt Solution (HBSS) at room temperature (RT) and then incubated with a minimal volume (enough to cover the area of flask) of 10% (v/v) trypsin in versene. An appropriate volume of FGM was added to neutralise the trypsin/versene and the cells were then divided accordingly.

2.3.2 27/12/M:4 (M49)

M49 is a stable cell line derived from BHK cells expressing the HSV-1 genes ICP4 and ICP27 (Thomas et al., 1999b). Cells were cultured in FGM containing 5% (v/v) tryptose phosphate broth, 800μg/ml of neomycin and 750μg/ml of zeocin. Cells were passaged as described before for BHKs (2.3.1).

2.3.3 C3H10T1/2 mouse fibroblasts

Mouse Fibroblast C3H/10T1/2 cells Clone 8 (ATCC #CCL-226) were maintained in DMEM medium containing 10% FCS. Cells were passaged as described in (2.3.1).

2.3.4 PC12 cells

Rat PC12 cells (ATCC #CRL-1721) were maintained in DMEM containing penicillin (100units/ml), streptomycin (100μg/ml), 10% horse serum (Gibco) and 5% foetal bovine serum (Gibco) on poly-L-lysine coated (0.1mg/ml) tissue flasks. Cells were passaged as described in (2.3.1).

2.3.5 293 cells

293-T cells were obtained from the European Collection of Animal Cell Cultures. Cells were cultured in full growth medium containing 10% FCS and passaged as described in (2.3.1).

2.3.6 Preparation of primary dissociated neural progenitor cultures

Time-mated rats were terminated by CO_2 asphyxiation followed by cervical dislocation. Following abdominal laparotomy, uterine horns were removed and collected in Hanks' balanced salt solution (HBSS, w/o Ca++ and Mg++, Gibco). The embryos were removed under aseptic conditions, collected in HBSS at room

temperature and crown-rump lengths measured to verify age. The heads were separated from the embryos and the skull and meninges removed using dissections scissors (number 5, DuMont). The CNS was exposed and the brainstem isolated. The mesencephalic flexure is deeply curved at this stage of development. To remove the mesencephalon a cut was made at the junction of the diencephalon and mesencephalon using the prominent diencephalic protuberance as a point of reference and another cut at the mesencephalic/metencephalic boundary. Using microscissors the ventral part of the mesencephalon was released by cutting along the tegumental midline through the ventricular opening. The dissected ventral mesencephalic tissue has a butterfly similar shape and the corners comprising tegmental tissue were trimmed off. Any attached meninges was removed and the explants pooled in HBSS on ice. The telencephalon was used to excise cortex by cutting through the ventricle from caudal to rostral and removing the cortical tissue from the ganglionic eminence. The striatum comprising medial and lateral ganglionic eminence was excised and cortical tissue trimmed off. Cortical and striatal explants were separately pooled in HBSS and stored on ice.

The HBSS was removed and the pooled explants were then incubated in 1.5ml trypsin (0.1% trypsin solution, Sigma) for 10min at 37°C. The excess trypsin was removed and the explants incubated in 1.5ml soyabean trypsin inhibitor (0.1% in HBSS, Sigma) for 5min at 37°C, prior to rinsing in DNAse (0.1%DNAseI in HBSS, Sigma). Any excess buffers were removed and the explants were resuspended in culture media (Neurobasal based media for primary neural progenitor cultures or DMEM/F12 based media for neurosphere cultures; 50μl per dissected embryo). Explants were then dissociated into a quasi-single cell suspension by gentle tritutration using 1000μl Eppendorf pipette tips. Viability was estimated using the trypan blue exclusion method and cell density calculated.

2.3.7 Neural progenitor cultures

Dissociated neural precursor cells (2.3.6) were plated at a density of $1x10^5$ viable cells/well on PLL/Laminin coated glass coverslips (2.3.10) in a total volume of 30μl. After attachment cells were overlayed with 500μl Neurobasal® medium containing B27 supplement (2% v/v), L-Glutamine (500μM), penicillin (100units/ml) and

streptomycin (100μg/ml). The medium in primary neural progenitor cultures was changed daily.

2.3.8 Neurosphere cultures

Dissociated rodent cortical and striatal cells were seeded at clonal density (20 viable cells/μl) into T75 flasks initially in a total volume of 10ml of defined serum-free proliferation medium (DMEM:HAMS-F12 at 3:1) supplemented with B27 (2% v/v), penicillin (100units/ml), streptomycin (100μg/ml) and fibroblast growth factor (20ng/ml, R&D). Dissociated mesencephalic rodent cells were plated at a density of 100 viable cells/μl in a total of 10ml proliferation medium. Only cultures to study the effects of heparin on viral transduction contained heparin (5μg/ml). Two days after seeding 10ml fresh proliferation medium were added to the neurosphere cultures. The medium of neurosphere cultures was changed every three days by replacing half of the medium with fresh proliferation medium. Neurospheres were passaged after 7-10 days by preparation of a quasi single cell suspension (2.3.6).

2.3.9 Preparation of a quasi-single cell suspension using Accutase™

Neurospheres were collected by brief centrifugation (1000rpm for 3min) and resuspended in 500μl Accutase™ followed by incubation at 37°C for 15min. Accutase™ was removed and neurospheres washed once in DMEM/F12 media and resuspended in 500μl differentiation medium. Neurospheres were triturated using a 1000μl pipette tip, the cell density determined by the trypan blue exclusion method (2.3.12) and plated on PLL/Laminin coated glass coverslips. After attachment dissociated neural precursor cells were overlaid with 500μl differentiation media.

2.3.10 Coating of glass cover slips

Sterile glass coverslips (Bellco) were placed into the wells of a 24 well plate and overlayed with 500μl of 0.1mg/ml Poly-L-Lysine (PLL) prepared in ddH_20. After incubation for more than 1hour at RT the PLL was removed and the coverslips rinsed with ddH_2O three times. The plates were dried under sterile environment and stored at

RT. Prior to use the coverslips were coated with laminin by placing 40μl of 50μg/ml laminin into the centre of the coverslip and incubation at 37°C for 30min. The laminin was removed and the spot washed three times with DMEM. For plating of cells the last wash was removed and the cells added in a total volume of 30μl. Cells were allowed to attach in a tissue culture incubator and subsequently overlaid with the appropriate medium.

2.3.11 Poly-HEMA coating of tissue culture plastic ware

For some neurosphere cultures tissue culture ware was coated with Poly-HEMA as required to prevent attachment to the tissue culture surface. Poly-HEMA is prepared at a final concentration of 12mg/ml in 95% ethanol. The tissue culture flasks were coated with 0.1ml/cm^2 of surface and allowed to dry O/N in a sterile environment.

2.3.12 Trypan blue cell exclusion method

Dissociated cells were diluted as required (1/10 to 1/100) in 0.4% Trypan blue/DEMEM (1:1) (T8154, Sigma). 10μl of the dilution was prepared in an improved Neubauer chamber (Hausser, USA) and four groups counted under a bright light microscope. Cells that did not include the dye were counted as viable cells. The cell concentration was determined accordingly.

2.3.13 Cell Line Storage

Cell stocks for long term storage were prepared by suspending the cells from one 175 cm^2 flask in freezing media A (DMEM supplemented with 20% FCS) followed by addition of an equal volume of freezing media B (DMEM supplemented with 40% FCS and 16% dimethylsulphoxide (DMSO)) and aliquoted into 1.5 ml cryovials. These were slowly cooled to -70°C, and then immersed in liquid nitrogen. The cells were recovered by rapidly thawing the contents of one cryovial and then transferred to a 25 cm^2 flask of pre-warmed medium. The medium was changed or the cells passaged the following day.

2.4 Virus construction and propagation

Hexamethylene *bis*-acetamide (HMBA) was added to the growth media at a final concentration of 3mM in order to induce immediate early gene expression in HSV-1 mutants with a mutated VP16 gene (McFarlane et al., 1992). M49 cells were used in all cases for virus generation and propagation.

2.4.1 Homologous recombination transfections

M49 cells were co-transfected with 5-10μg of linearised plasmid DNA and 10-30μg of purified viral DNA per transfection. The transfections were based on the calcium phosphate method as described previously (Stow and Wilkie, 1976). Cells were prepared to be 70-80% confluent. Two tubes were set up, A and B. Tube A contained 31 µl 2 M $CaCl_2$, 10 µg plasmid DNA and 20 µg herring sperm DNA. Tube B contained 400 µl HEBES transfection buffer. HEBES buffer contained 140 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM D-glucose, 20 mM Hepes, pH 7.05 with NaOH and was filter sterilised with a 0.2 µm filter and stored at 4°C. The contents of tube A were carefully mixed by gentle pipetting and then added to tube B in a drop-wise manner with gently mixing. The mixture was then left for 20-40 min to allow the DNA to precipitate. Media was removed from the cell monolayer and the precipitated DNA mixture was then added (one transfection per well) and incubated for 7 hours prior to DMSO shocking. To DMSO shock, media was removed from the cells, and the cells were washed twice with 2 ml of FGM. 1 ml of ice-cold 20% (v/v) DMSO in HEBES transfection buffer was added to the cells and left for 90 seconds. The DMSO solution was removed and the cells were washed twice with 2 ml of FGM. A final volume of 2 ml of FGM was added to the cells and then these were incubated at 37°C/5%CO_2 for 3-5 days until complete CPE was observed. The cells were then harvested and freeze-thawed. The harvested cells were titred (2.4.2) and the efficiency of the recombination determined by assaying for loss of reporter gene expression.

2.4.2 Viral titration

Serial ten fold dilutions of a virus suspension (either harvested from homologous recombination or from a pure stock) were prepared in DMEM without FCS and plated onto 80% confluent wells of M49 cells in a total volume of 500μl. The virus was allowed to adsorb for 60 min at 37°C/5%CO_2 and then the monolayers were overlaid with 2 ml of a 1:2 (v/v) mixture of 1.6% carboxymethylcellulose (CMC):FGM supplemented with 3 mM HMBA. The cells were then incubated for a further 48 hours at 37°C/5% CO_2 and the number of plaques were counted in order to determine the titre of the virus in plaque forming units (pfu)/ml.
Cells expressing green fluorescent protein (GFP) required no pre-treatment and were visualised directly under an inverted fluorescent microscope at a wavelength of 520nm.

2.4.3 Purification of viral recombinants by plaque selection

Titred transfection mixes were visualised under an epifluorescent microscope for viral plaques with the lack of GFP expression. The identified plaques were picked from the cell monolayer using a P20 Gilson micropipette (set at 3μl). Selected plaques were transferred into 100μl SFM and frozen at -80°C. The plaque suspension was thawed and 10μl and 90μl used to infect wells of a 6 well plate containing M49 cells at 80% confluency. Infection was performed as described in 2.4.2 and the cells were then incubated for a further 48 hours at 37°C/5%CO_2. The plaque purification process was repeated until a pure population was obtained. When this was achieved a single plaque was used to infect one well of a 6 well plate and incubated until CPE was detected. The whole well was then harvested and its titre determined. This was then used as a master stock (MS) for large-scale propagation of the recombinant virus.

2.4.4 High titre stock production

Approximately 300μl of MS was used to infect 90% confluent M49 cells in 80cm^2 flasks. After incubation for 1hour at 37°C/5%CO_2 cells were overlaid with FGM containing 3mM HMBA. Cells were harvested when complete CPE was observed.

The virus was grown in 175cm^2 flask until enough virus was obtained to infect 10 x 224mm^2 plates of M49 grown to 90% confluency. Each plate was infected with 5x10^{6}pfu of virus stock suspension in a total volume of 50ml FGM supplemented with HMBA. Cells were harvested by freezing at 80°C, followed by thawing and cell suspension of the plates was pooled. Cells were centrifuged at 3500rpm for 45min to remove cell debris. The supernatant was then sequentially filtered through a 5.0μm and a 0.45μm filter. To pellet the virus particles, the supernatant was spun at 12000rpm for 2hr at 4°C. The supernatant was removed and a small volume of SFM media added (50μl per 250ml cell suspension). The virus pellet was resuspended by shaking on an orbital shaker O/N at 4°C. 25μl and 50μl aliquots of the resuspended virus were stored in liquid nitrogen. The titre of the resuspended virus was determined (2.4.2).

2.5 Immunolabelling

2.5.1 Western blotting

Cells were washed twice with 1x PBS and harvested in 100μl protein loading buffer (7M Urea, 0.1% $NaH_2PO_4H_20$, 1% SDS, 100mM DTT, 0.1% brombhenol blue pH7.2) and samples were frozen down at -20°C. Before loading on an SDS-polyacrylamide gel the samples were boiled at 95°C for 5min, cooled down on ice, briefly spun down and stored on ice until loading.

2.5.1.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gels were prepared and run in a vertical gel electrophoresis system according to the method of Laemmli (Laemmli, 1970). The composition of stacking and resolving gels can be seen in 2-1. The resolving gels used were of 15% for samples of FGF8, FGF2, ShhN and GFAP or 12% for Nurr1 and TH. Resolving gels was overlaid with water saturated n-butanol, the solvent removed and subsequently the gel surface washed with dH_20 and dried before it was overlaid with

the stacking gel. Gels were run at constant current (30mA/gel) with variable voltage in 1x running buffer (25mM Tris, 250mM glycine and 0.1% (w/v) SDS, pH8.3). Gels were run until the protein of interest was approximately in the middle of the gel, as determined by the migration of the coloured molecular weught marker.

Resolving Gel	**12% gel**	**15% gel**
dH_2O	16.5 ml	11.5 ml
30% acrylamide mix*	20.0 ml	25.0 ml
1.5M Tris (pH 8.8)	12.5 ml	12.5 ml
10% SDS	0.5 ml	0.5 ml
10% ammonium persulfate	0.5 ml	0.5 ml
TEMED	0.02 ml	0.02 ml

Stacking Gel	**5% gel**
dH_2O	6.8 ml
30% acrylamide mix*	1.7 ml
1.0M Tris (pH 6.8)	1.25 ml
10% SDS	0.1 ml
10% ammonium persulfate	0.1 ml
TEMED	0.01 ml

Table 2-1: Composition of stacking and resolving gels used in SDS-PAGE. Acrylamide mix = acrylamide-bis acrylamide (30:1.5).

2.5.1.2 Transfer of proteins to nitrocellulose membranes (western blot)

Proteins were transferred from the SDS-polyacrylamide gel to Hybond-C nitrocellulose membranes by a wet transfer method, based on that previously described. Briefly, the SDS-PAGE gel and the nitrocellulose membrane were pre-soaked in transfer buffer (50mM Tris, 180mM glycine, 0.1% (w/v)SDS and 20% (v/v) methanol) and then sandwiched between sheets of 3MM Whatman paper and a Trans-Blot™ cell (BioRad) assembled according to the manufacturer's instructions. Transfer was carried out at 10V O/N and 4°C.

2.5.1.3 Immunodetection

Throughout the protocol the blots were shaken constantly on an orbital shaker. Following transfer, the membranes were rinsed three times for 5 min in T-PBS (1x PBS, 0.1% Tween-20) and blocked in 5% (w/v) skimmed milk powder in T-PBS for at least 1 hour. The membrane was then incubated in primary antibody diluted in blocking buffer containing 0.02% (w/v) Na-Azide for 1 hour at RT or O/N at 4°C. The primary antibody dilutions were as follows: FGF8 (1:400), FGF2 (1:200), ShhN (1:400), Nurr1 (1:200), TH (1:150), GFAP (1:500), GAPDH (1:500). Unbound antibody was removed by washing the membranes for 3x10min in T-PBS. The membrane was then incubated with secondary antibodies (all from DAKO horseradish peroxidase (HRP) conjugated; 1:1000 in blocking buffer) for 1 hour at RT. Unbound antibody was removed by washing as before. The bound HRP was then detected using an enhanced chemiluminescence system (ECL™) according to manufacturer's instructions. The membrane was then exposed to X-ray film, from 1sec to 15min, depending on the signal intensity.

2.5.2 Immunocytochemistry (ICC)

Immunocytochemical stainings were performed after fixation of cells on the glass coverlsips. All washing steps were carried out on an orbital shaker.

2.5.2.1 Fixation of cells

The medium was removed and the cells rinsed with 1x PBS. 4% (w/v) paraformaldehyde (PFA) in 1x PBS was freshly prepared, the pH adjusted to 7.4 and stored for up to one week at 4°C. Cells were fixed in ice-cold 4% PFA for 30min at RT. Coverslips were washed three times with 1x PBS and stored in PBS at 4°C until the staining was performed.

2.5.2.2 Immunocytochemical staining

Cells were blocked in 5% (v/v) normal goat serum (NGS) in 0.1% (v/v) TritonX-100 in PBS for 1h at RT. Antibody incubations were carried out as "floating drop" technique: a small volume (60-100μl) of the appropriate antibody was placed as a drop on Parafilm™ in a humid chamber and the coverslip with the cells facing down carefully placed on this drop of antibody. Primary antibodies were prepared in 5% NGS at the appropriate dilution as decribed in Table 2-2 and the cells incubated for 1h at RT. The coverslips were removed, placed into a 24 well plate and washed 3x 10min with 1x PBS. Appropriate secondary antibodies were made up according to Table 2-2 in 5% NGS and cells incubated as described before as floating drop for 1hr at RT. Coverslips were washed 3x for 10min in 1x PBS. As required cell nuclei were stained by incubation with Hoechst (1:10,000 in PBS) or DAPI (1:10,000 in PBS) for 10min at RT and rinsed 3x with 1x PBS. After the last wash, the coverslips were removed and mounted on a small drop (40μl) of Antifade™ Prolong mounting media. The edges of the coverslips were sealed with nail varnish and the specimen protected from light and stored at 4°C.

Alterations to this protocol were as follows: (i) in case that the secondary antibody was not raised in goat, all solutions were made up in serum of the same species as the secondary antibody was raised in. (ii) in case of immunolabelling for galactocerebroside (GalC) or oligodendrocyte marker O4 the detected epitopes are surface molecules and thus, cells were not permeabilsed with 0.1% TritonX-100. The incubation with the O4 primary antibody was carried out at 37°C according to manufacturer's instructions. (iii) multiple labellings were performed sequentially to avoid cross-reactions between antibodies.

2.5.2.3 Sample preparation for BrdU staining

Immunolabelling for BrdU requires unraveling of the DNA since the antibody recognizes single stranded DNA. Fixed cells were incubated with 500μl 2N HCL per well for 20min at 37°C for acid denaturation. The acid was removed and 500μl 0.1M Na-Borate in ddH_2O, pH8.5 added per well to neutralize for 10min at RT. The

neutralization step was repeated once and thereafter, the cells were washed 3x for 10min in 1x PBS at RT. Blocking and immunocytochemical staining were performed as described in 2.5.2.2. Double labelling of BrdU and an additional marker was performed sequentially with the staining for BrdU first.

2.5.3 Immunohistochemistry (IHC)

2.5.3.1 Tissue preparation

Animals were transcardially perfused with ice-cold 4% (w/v) paraformaldehyde (PFA) in 0.1M phosphate buffer pH 7.4, the brains removed and post-fixed for one hour or O/N depending on the quality of the perfusion. The brains were then protected in 30% (w/v) sucrose in 0.1M phosphate buffer pH 7.4 containing 0.02% sodium azide until they have sunk. Tissue was cut on a freezing microtome in sections between 20-100μm and the sections stored in PBS containing 0.02% Na azide.

2.5.3.2 Immunohistochemical staining

Throughout the protocol incubations were performed free floating, shaking on an orbital platform. For BrdU IHC the tissue was prepared as described in 2.5.2.3. Sections were blocked in 5% NGS in PBS containing 0.3% TritonX-100 for 2h at RT. Tissue was transferred into primary antibodies made up in 5%NGS in PBS and incubated O/N at 4°C. The antibodies were diluted according to Table 2-2. Sections were washed 3x 10min in PBS and transferred into the secondary antibody for 1h at RT. Tissue was washed 3x 10min in PBS and mounted on subbed slides (BDH, UK). All double labellings were performed sequentially. The specimen were air-dried and mounted in antifade mounting media.

2.5.3.3 X-Gal staining for detection of β-galactosidase

Sections were incubated in X-gal solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6.6H_2O$, 1 mM $MgCl_2$, 150 µg/ml X-gal in DMSO in 1x PBS) at 37°C for at least 2hr. The X-gal stain was then removed and sections washed three times in PBS.

Primary antibody					Corresponding secondary antibody			
description	**class**	**company**	**ICC**	**IHC**	**description**	**company**	**ICC**	**IHC**
rabbit anti-tyrosine hydroxylase	p IgG	Pel-Freez	1:150	1:150	Alexa Fluor 546 goat anti-rabbit	Molecular Probes	1:1000	1:500
rabbit anti-GFAP	p IgG	Dako	1:500	1:500	Alexa Fluor 546 goat anti-rabbit	Molecular Probes	1:1000	1:500
mouse anti-NeuN	m IgG1	Chemicon	n/a	1:500	Alexa Fluor 546 goat anti-mouse IgG (H+L) HC	Molecular Probes	n/a	1:500
mouse anti-TuJ1	m IgG2b	Sigma	1:100	n/a	Alexa Fluor 488 goat anti-mouse IgG2b	Molecular Probes	1:1000	n/a
					Alexa Fluor 546 goat anti-mouse IgG (H+L) HC	Molecular Probes	1:1000	n/a
					Rhodamine goat anti-mouse IgG (H+L)	SB	1:200	n/a
mouse anti-O4	m IgGM	Chemicon	1:50-1:100	n/a	Alexa Fluor 350 goat anti-mouse IgM	Molecular Probes	1:500	n/a
mouse anti-GalC	m IgG3	Chemicon	1:20	n/a	Alexa Fluor 350 goat anti-mouse IgG3	Molecular Probes	1:500	n/a
					TRITC donkey anti-mouse IgG			
rat anti BrdU	m IgG2a	abcam	1:100	1:100	Alexa 488 goat anti-rat IgG	Molecular Probes	1:500	1:1000
mouse anti-nestin	m IgG1	Chemicon	1:200	1:200	Alexa 546 goat anti-mouse IgG1	Molecular Probes	1:1000	1:500
					Fluorescine goat anti-mouse IgG (H+L)	SB	1:200	n/a
					Alexa Fluor 546 goat anti-mouse IgG (H+L) HC	Molecular Probes	1:500	1:500
Abbreviations:	n/a	not tested						
	SB	Southern Biotechnology						
	HC	highly cross absorbed						

Table 2-2: Antibody dilutions for ICC and IHC.

2.6 *In vivo* work

All experiments were carried out in accordance with the UK Animal Scientific Procedures Act 1986.

All animals were obtained from breeding colonies within the Department of Biological Services, UCL or from Harlan Labaratories (Oxon, UK and Madison, Wisconsin, USA). All animals were female Sprague-Dawley rats and weighed at least 220g. The age of time-mated animals was assessed with the vaginal plug as E=0.

2.6.1 Stereotaxic injections

For all CNS injections a Kopf stereotaxic frame (dual small animal stereotaxic, Kopf Instruments, USA) was used and coordinates established according to a stereotaxic rat brain atlas (Paxinos and Watson, 1997). The head of the anaethetised rat was positioned into the frame, and a sagital incision was made to expose the skull. Tissue was removed and bregma and lambda points marked. Bregma was defined as the point of intersection of the sagittal suture with the curve of best fit along the coronal suture. When the two sides of the coronal suture met the sagittal suture at different points, bregma usually falls midway between the two junctions. The incisor bar adjusted until the heights of lambda and bregma were equal (flat-skull position). The tip of the needle was positioned at Bregma, the appropriate anterior-posterior and medial-lateral coordinates calculated and the injection site marked. A whole was drilled through the skull without damaging dura. The needle was lowered until the tip just touched the tissue surface. The dorsal-ventral coordinates were calculated and the needle accordingly lowered. Injections were carried out using an automatic microinjector (World Precision Instruments, USA) with a flow rate set at 0.5-1μl/min. Following injection, the needle was left in place for an additional 5min to allow the injectate to diffuse from the needle tip. The needle was then removed within 3min. The wound was closed using metal clips and the animals allowed to recover.

2.6.2 *In vivo* BrdU injections

5-bromo-2'-deoxyuridine (BrdU) (B5002, Sigma, UK) was freshly prepared. BrdU was solubilised at 500mg/ml in DMSO and diluted in sterile 0.1M Tris/HCl pH7.4 to a final concentration of 50mg/ml. Animals were injected intra peritoneal (i.p.) with 100mg/kg per day.

2.7 Detection of fluorescence

GFP expression was visualized under UV light (500nm). Alexa Fluor® 350 dye was visualised at an absorption of 350nm, Alexa Fluor® 546 and TRITC conjugated dyes at 560nm, Alexa Fluor® 488 and FITC conjugated dyes at an absorption of 500nm.

2.8 Statistic analysis

Every experiment was repeated at least two times in duplets cultures. For each experiment a minimum of 10 fields per 13mm coverslip were randomly selected. Statistic analysis was performed using the appropriate statitistical test. Statistical comparisons were made by ANOVA when more than three groups were involved and the data followed a Gaussian distribution. Data were expressed as means ± standard error of the means (SEM). All statistics were performed using the Prism software package Version 3.02 (Graphpad Software Inc, CA, USA).

CHAPTER 3:

EVALUATION OF HERPES SIMPLEX VIRUS 1 (HSV-1) BASED VECTORS FOR DELIVERING RECOMBINANT GENES TO NEURAL PRECURSOR CELLS *IN VITRO* AND *IN VIVO*

3.1 Introduction

Multiple approaches have been unveiled for the use of neural precursor cells. Despite their therapeutic potential to replace various neural cell types lost or damaged in human diseases (Bjorklund and Lindvall, 2000), they offer a unique system to study neural development (Bhattacharyya and Svendsen, 2003), model neurological diseases (Jakel et al., 2004) or as assays to test pharmacological components (Calhoun et al., 2003). However, any of these studies may require ectopic gene expression involving the introduction of recombinant transgenes into neural precursor cells. Replication deficient viruses have become a powerful tool for gene targeting to neural stem cells and most commonly used vector systems are adenoviruses (Yoon et al., 1996; Corti et al., 1999; Hughes et al., 2002; Falk et al., 2002), adeno-associated viruses (Davidson et al., 2000; Wu et al., 2002), retroviruses (Falk et al., 2002; Kageyama et al., 2003), and lentiviruses (Englund et al., 2000; Asahara et al., 2000; Buchet et al., 2002).

This chapter evaluates the potential use of disabled HSV-1 as a gene delivery vector to neural precursor cells *in vitro* and *in vivo* and emphasizes the effects of viral transduction on cellular integrity of the neural stem cell.

Neural precursor cells isolated from the developing CNS can be either maintained as a mixture of primary neurons and neural progenitor cells or expanded as neurosphere cultures (Gage et al., 1995). Based on these two *in vitro* models, we compared two replication-deficient HSV-1 viruses to find an adequate vector for *ex vivo* gene transfer approaches. The optimal vector system delivers recombinant genes efficiently at low viral doses and does not cause cytotoxic effects or interference with the function of the transduced precursor cell.

Previously our group has developed a multiple immediate-early gene deficient HSV-1 vector deleted for ICP27, ICP4, and ICP34.5 with an inactivating mutation in VP16 (Lilley et al., 2001), termed 1764/27-/4-. This viral backbone was chosen as it efficiently transduces neurons of the CNS *in vitro* and *in vivo* without any cytotoxic site effects reported (Lilley et al., 2001). Further vector development has led to a

vector containing ICP27, ICP34.5 and with a promoter cassette containing the woodchuck hepatitis virus posttranscriptional regulatory element (wpre). ICP27 and ICP34.5 were shown to be anti-apoptotic (Chou and Roizman, 1992; Aubert et al., 1999; Zachos et al., 2001) and the woodchuck response element increases transgene expression through a combination of stabilisation of RNA transcripts and facilitation of their cytoplasmic export as demonstrated for retroviral (Zufferey et al., 1999) and adenoviral (Xu et al., 2003) constructs. In primary cortical neuronal cultures this less disabled vector, referred to as RL1+/27+/4-, showed increased transduction efficiency compared to previous vectors (Li, personal reference). However, these previous studies did not focus on gene delivery to primary neuronal cultures from other regions of the developing CNS and no data were available on transduction efficiency to mesencephalic or neurosphere cultures that are particularly relevant for this study.

Under the given rationale, the first part of this chapter compared gene delivery efficiency and cytotoxicity of these two viral backbones in primary cortical, striatal and mesencephalic progenitor cultures. A comparison to other HSV-1 based vectors is complicated by the use of different disablements of the viral constructs, and the preparation of tissue from different neurogenic regions at various gestational stages. In other reports e.g. gene delivery efficiency to cortical (Anderson et al., 2001), striatal (Freese and Geller, 1991; Aboody-Guterman et al., 1997) and mesencephalic neuronal cultures (Costantini et al., 1999) varied between 2% and 100%, indicating the variance in the experimental conditions.

In the second part of this chapter neurospheres were used as a model to study gene delivery and vector cytotoxicity to neural stem cells. Viral transduction may affect the cellular characteristic of the neural stem cell, aforementioned as self-renewal capacity and multipotency. Therefore, we characterized the antigenic identity of the transduced neural precursor cell and determined effects of HSV-1 infection on the differentiation potential into the different neural lineages.

In the third part of this chapter gene delivery to endogenous neural stem of the adult subventricular zone (SVZ) was studied. Other groups have shown before that replication-competent HSV-1 infects ependymal cells of the SVZ (Kesari et al.,

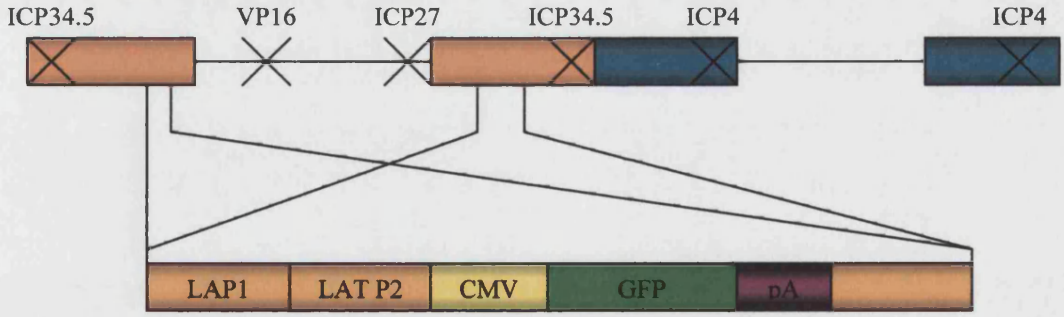
1998), but did not further characterize the antigenic character of the transduced cells. As astrocytes were identified as the potential neural stem cells within the SVZ (Doetsch et al., 1999), the work presented here focused on the antigenic identity of the transduced ependymal and subependymal cells. Neuronal precursors born in the SVZ migrate to the olfactory bulb where they differentiate into neurons (Lois and Alvarez-Buylla, 1994). Migration of transduced precursor cells was monitored by reporter gene expression in the olfactory bulb.

3.2 Materials and Methods

3.2.1 Viral constructs and promoters used

Vectors tested were based on wild type HSV-1 strain 17syn+ (Brown et al., 1973). The term "1764" describes a virus with the *in*1814 mutation in the gene encoding VP16 and with genes encoding ICP34.5 and ORF P completely deleted (between nt 124945 and 125723). The vector 1764/27-/4- is deleted for ICP27 (nt 113273 and 116869 comprising UL54, -55, and-56) and both copies of ICP4 (Lilley et al., 2001). The promoter cassettes pR19hGFP or pR19LacZ were recombined into the endogenous LAT regions between the two BstXI sites (nt 120220 and 120408) (Lilley et al., 2001). The resulting virus is referred to as 1764/27-/4-pR19hGFP or 1764/27-/4-pR19LacZ, respectively (Figure 3-1). Based on this vector the genes encoding for ICP27 and ICP34.5 were inserted back into the viral genome and a reporter gene cassette constructed containing the woodchuck hepatitis virus posttranscriptional regulatory element (wpre), resulting in the vector RL1+/27+/4-pR19hGFPwpre (Li, unpublished data).

1764/27-/4-/pR19hGFP (Lilley et al., 2001)



RL1+/27+/4-/pR19hGFPwpre

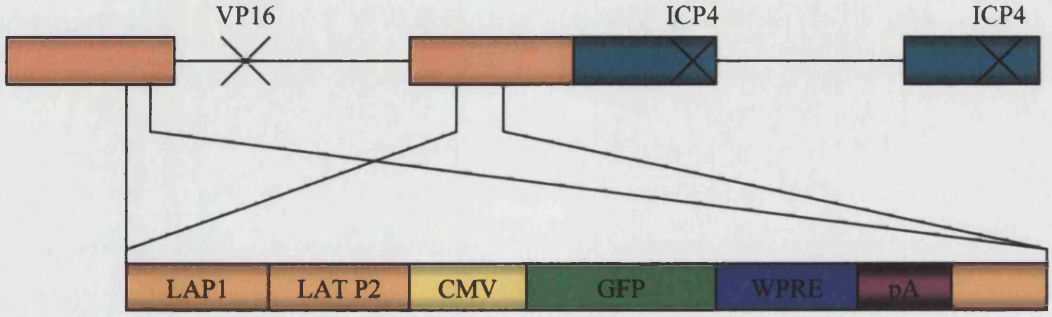


Figure 3-1: Replication incompetent vector backbones and promoter cassettes.

3.2.2 Viral infection of primary neurons and neural progenitor cells

Primary neural progenitors (2.3.6) were cultured on substrate coated glass coverslips (2.3.10) in 24 well plates. For viral transduction the media was removed and cells were overlaid with 200μl Neurobasal® media per well containing the appropriate amount of virus and incubated for one hour at 37°C/5%CO_2 in a standard tissue incubator. During infection the plate was tilted every 10-15min. Following infection the virus containing media was removed and replaced by Neurobasal® media containing B27 supplement (2% v/v, Gibco), L-glutamine (500μM), penicillin (100units/ml) and streptomycin (100μg/ml). The medium in primary neural progenitor cultures was changed daily.

3.2.3 Assessment of cell concentration and viral infection of rodent and human neurospheres

For gene delivery studies cell concentration was assessed by selecting 100 medium sized neurospheres (about 300μm diameter) under a dissecting microscope in a flow hood. The neurospheres were pooled in a 1.5ml tube and settled by short centrifugation (1000rpm for 2 min). The supernatant was removed and the spheres resuspended in 500μl Accutase™ for cell dissociation. After incubation for 15min at 37°C, spheres were briefly centrifuged (1000rpm for 3min) and the Accutase™ removed. Neurospheres were resuspended in 200μl DMEM and triturated into a single cell suspension using a 200μl pipette tip. The cells were counted using the trypan blue exclusion method (2.3.12) and the cell concentration determined as cells per neurosphere.

For viral infection neurospheres of the same size were selected and pooled in a 1.5ml test tube. Excess medium was removed and the neurospheres resuspended in 50μl DMEM/F12 media containing the appropriate amount of virus. During infection the spheres were incubated for one hour at 37°C and the tubes finger vortexed every 15min. The neurospheres were briefly centrifuged (1000rpm for 3min), the virus containing media removed, resuspended in appropriate media and transferred into T25 flasks containing 10ml of growth or differentiation medium

(DMEM/F12 containing B27). Medium of infected neurospheres was changed every three days by replacing half of the medium by fresh medium.

For all other infections of neurospheres, cell concentration was determined by dissociation of an aliquot of the neurosphere suspension using Accutase™ and trituration as described above. After determination of the cell concentration using the trypan blue exclusion method (2.3.12), the virus amount required for infection of an appropriate number of neurospheres was calculated.

Human foetal tissue (8-12 weeks postconception) was used in this study collected following routine termination of pregnancy. The methods of collection conform to the arrangements set out by the Department of Health in the United Kingdom. Human neurospheres were expanded over long time in (DMEM:HAMS-F12 at 3:1) supplemented with N2 (1% v/v), penicillin (100units/ml), streptomycin (100μg/ml) containing EGF (20ng/ml, R&D) and LIF (10ng/ml, Chemicon). Human neurospheres require a cell-cell contact for long term expansion and were passaged using a unique chopping method as previously described (Svendsen et al., 1998). Assessment of cell concentration and viral infections of human neurospheres were performed as described for rodent neurospheres.

3.2.4 Cell migration assay

To monitor the migration and differentiation of single precursor cells, whole neurospheres were plated on poly-L-lysine/Laminin-coated glass cover slips in a total volume of 30μl in a 24 well plate. After one hour incubation in a humidified incubator (37°C, 5%CO_2), the neurospheres had attached to the surface and short processes emerged from the neurospheres. The cultures were overlaid with 500μl appropriate proliferation or differentiation medium.

3.2.5 Stereotaxic injections

For intraventricular injections $5x10^6$ PFU of RL1+/27+/4-pR19hGFPwpre in a volume of 5μl were injected bilaterally into each lateral ventricle of adult female

Sprague-Dawley rats (250g-280g, Harlan, UK) (n=3). Using a Kopf Instruments stereotaxic frame, the rats were injected at the following coordinates relatively to Bregma: AP –0.3mm, ML ±1.2mm, DV -3.6mm (Paxinos and Watson, 1997). Three days after injection rats were transcardially perfused with 4% paraformaldehyde, the brains removed and conserved as described in 2.5.3.1. The brains were cut into 40μm coronal sections. Immunohistochemical staining was performed as described in 2.5.3.

For injections into the SVZ $5x10^5$ PFU of 1764/27-/4-/pR19LacZ in a total volume of 5μl were stereotaxically injected unilaterally into the dorsocaudal horn of the lateral ventricle of female adult Sprague-Dawley rats (250g-280g, Harlan UK) (n=3) at the following coordinates relatively to Bregma: AP -0.3mm, ML-2.0mm, DV -3.1mm (Paxinos and Watson, 1997). Three days after viral injection the animals were perfused, the brains removed containing the entire olfactory bulb and sliced into 60μm coronal sections. The sections were stained for LacZ expression as described in 2.5.3.3.

3.2.6 Transplantation of neurospheres

Neurospheres derived from E14 ventral mesencephalon were expanded for 10 days as neurospheres. Neurospheres were infected with RL1+/27+/4-pR19hGFPwpre at an m.o.i. of 1 and maintained for another two days in differentiation conditions. An aliquot of the transduced neurospheres were plated on poly-L-lysine/laminin coated glass coverslips and stained for TH expression as described in 2.5.2. Before transplantation an aliquot of the neurosphere culture was removed and the cell concentration determined using trypan blue exclusion method. The neurospheres were diluted in DMEM/F12 media to a final concentration of 10,000 cells/5μl and aliquots of spheres were prepared individually for each animal prior to transplantation. Adult female Sprague Dawley rats (250-300g) (n=3) received unilaterally two grafts of 10,000 cells into the left striatum. Using a stereotaxic frame the whole neurospheres were injected with a 28"gauge needle attached to a 25μl Hamilton syringe at the following coordinates: AP +1.6mm, ML +2.4mm, and AP +0.2mm, ML +3.4mm, DV -4.5mm (Paxinos and Watson, 1997). The animals were perfused with 4% paraformaldehyde three days after injections, the brains removed and cut into 60μm

coronal sections. Immunohistochemical stainings were performed as described in 2.5.3.

3.3 Results

3.3.1 HSV-1 as a gene delivery vector to primary neurons and neural progenitor cells of the CNS

The first part of this chapter considered gene delivery to primary neurons and neural progenitor cells using replication deficient HSV-1 vectors. The population of fate restricted primary cells was characterized and viral gene delivery validated in terms of transduction efficiency and effects on neuronal cell loss.

3.3.1.1 Characterization of neural progenitor cells

Primary neurons and neural progenitor cells used in this study were isolated from three distinct neurogenic regions within the neuroepithelium of the developing rat CNS: cortex, striatum (comprising medial and lateral ganglionic eminences) and ventral mesencephalon. The aim of this first section was to characterize the cultures regarding their neural cell types.

The tissue was prepared from time-mated E14 Sprague-Dawley embryos and dissections were performed as described before (Dunnett and Bjorklund, 1992) (Figure 3-2). The explants were dissociated into a quasi-single cell suspension by trypsin treatment and trituration. Cell suspension preparation is a crucial step for setting up primary neuronal and neurosphere cultures. The physical process of cell suspension preparation from termination of the animal, embryo removal, tissue dissection, through enzyme treatment and dissociation, causes cellular stress resulting in necrosis and apoptosis (Branton et al., 1998). This cell damage and neuronal loss, which is particular evident for mesencephalic cultures (Fawcett et al., 1995), was also observed in this study when cell viability was assessed by trypan blue exclusion assay of the trypsin treated and triturated cell suspension. About 10% of the cells incorporated the dye and these damaged cells were not included when the cell density was determined prior to plating of primary cells or seeding of neurosphere suspension cultures. However, these cells may bind viral particles and it has to be considered that this may effect determination of gene delivery efficiency.

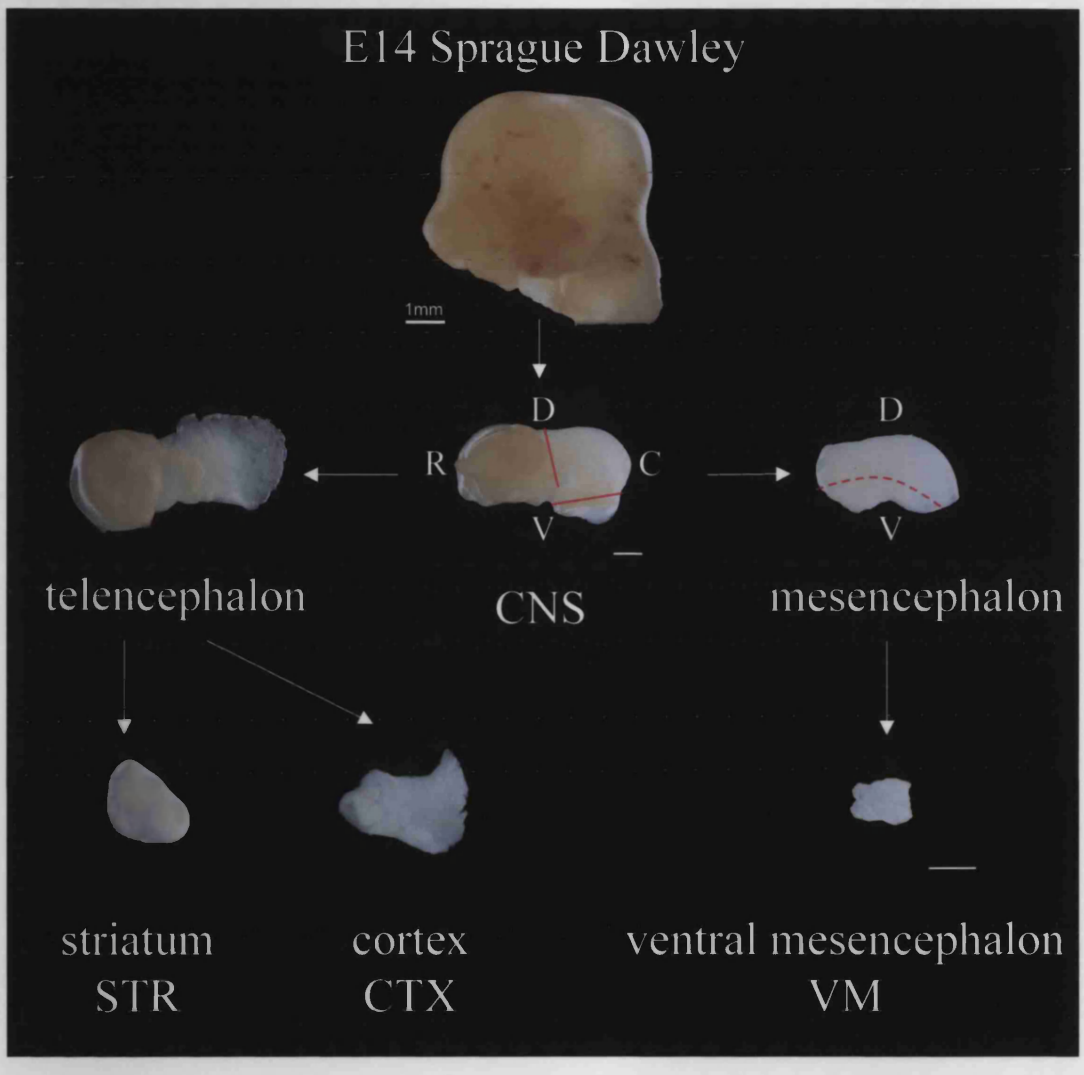


Figure 3-2: Isolation of primary tissue. E14 time-mated (plague = E0) Sprague-Dawley rats (Harlan, UK) were sacrificed and the embryos removed. The CNS was exposed and the mesencephalon released by dissecting along the forebrain/midbrain boundary and the midbrain/hindbrain groove (the red lines indicate the rostral and caudal incisisons, and the dotted line the incision made to isolate the ventral mesencephalon). Striatal (medial and lateral ganglionic eminence) and cortical tissue were isolated from the telencephalon. The tissue shown here was fixed in 4% paraformaldehyde overnight prior to photography. D=dorsal, V=ventral, R=rostral, C=caudal. Scale bar represents 1mm.

The dissociated cells were plated on poly-L-lysine (PLL)/laminin coated glass coverslips and maintained for seven days in serum-free Neurobasal™ media complemented with B27. Serum-free culture conditions were chosen to avoid any effects from undefined serum components. It has been reported previously that primary mesencephalic neurons can be maintained in Neurobasal™ media (Cheung et al., 1997) and neuronal survival increased by supplementing with B27 (Svendsen et al., 1995). The cultures followed a time dependent change regarding the amount of neurons and the morphology of the cells that also differed with the origin of the tissue (Figure 3-3). Generally, cells showed characteristics of immature neuroblasts on the first day in culture with a small oval cell body and two short processes. With continuous culture under serum-free conditions, the neural progenitors matured into neurons with a larger soma, longer and branched processes and distinctive phenotypes depending on the region the tissue was derived from. After three days in culture cortical neurons had predominantly large and polygonal cell bodies with multiple unbranched processes, while striatal neurons had smaller, oval shaped cell bodies with two to three long, unbranched processes. Striatal neural cells often formed spherical aggregates at later culture time points. Mesencephalic derived primary neurons can be classified as GABAergic and dopaminergic neurons (Hanaway et al., 1971; Altman and Bayer, 1981) with a large polygonal cell body. Dopaminergic neurons characterized by the expression of TH were found to be slightly larger than TH negative cells with a mostly pyramidal cell shape bearing typically two to five thick processes that gave rise to relatively sparse arborisation. Primary mesencephalic cultures contained a significant amount of cell debris on 1DIV compared to cortical and striatal cultures as a result from increased cell death during tissue preparation (Emgard et al., 2002), but appeared to recover within the first three days in culture. The cell density also affected the state of mesencephalic cultures. Low density cultures (less than $5x10^4$ cells per well) were difficult to maintain, a phenomenon that has also been described by other groups (Dal Toso et al., 1988).

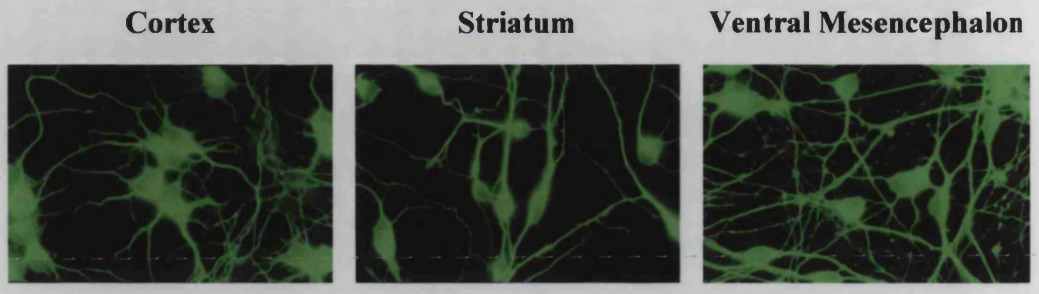


Figure 3-3: Distinct morphologies of neurons derived from different regions of the developing neuroepithelium. Neural progenitor cells from the developing cortex, striatum and ventral mesencephalon were plated on PLL/Laminin coated coverslips and maintained for three days in serum-free media. Cells were fixed with 4%PFA and stained for the neuronal marker TuJ1. Cortical neurons had a large, polygonal cell body, while striatal neurons were smaller and oval shaped with predominantly two long processes. Mesencephalic neurons were often polygonal bearing three to four processes. Pictures were taken at 100x magnification.

Cortex

DIV	TuJ1 (%)	nestin (%)	TH (%)
3	58.96±5.21	42.26±2.73	n/a
7	67.52±3.80	20.10±2.08[a]	n/a

Striatum

DIV	TuJ1 (%)	nestin (%)	TH (%)
3	87.92±3.83	7.70±1.62	n/a
7	85.47±3.49	8.11±1.85	n/a

Ventral Mesencephalon

DIV	TuJ1 (%)	nestin (%)	TH (%)
3	55.53±2.34	52.03±4.33	17.57±2.00
7	61.90±3.62	35.14±2.38[a]	10.60±2.88

Table 3-1: Composition of neural progenitor cultures. Cortical, striatal and mesencephalic tissue from E14 rats was plated at a density of $1x10^5$cells per well on PLL/Laminin coated coverslips. The cultures were maintained in serum-free Neurobasal™ medium supplemented with B27 for 3 or 7 days *in vitro*. Cells were fixed with 4%PFA and stained for the neuronal marker TuJ1, the neurofilament marker nestin or the dopaminergic marker TH. Data are presented as percentage of total cell number.

[a] significant decrease in the amount of nestin positive cells in cortical ($p<0.001$ in two tailed t test, n=3 independent experiments) and mesencephalic progenitor cultures ($p< 0.003$, n=3 independent experiments).

The primary cultures were characterized on 1 and 3 DIV by cell lineage specific markers for TuJ1 positive neurons (Banerjee et al., 1990), GFAP positive astrocytes (Hansen et al., 1989), O4 positive oligodendrocytes (Sommer and Schachner, 1981) and nestin positive undifferentiated neural progenitor cells (Hockfield and McKay, 1985) (Figure 3-4).

After three days in culture most of the striatal cells were identified as neurons, while only about half of the population of cortical and mesencephalic cells were positive for the neuronal marker TuJ1. As shown in Table 3-1, cortical and mesencephalic cultures contained a high proportion of undifferentiated progenitor cells positive for the neurofilament marker nestin. Although the culture conditions were free of any exogenous growth factors and thus, support only differentiation but not proliferation of the progenitors, the data suggest that these cultures had not fully matured at this early stage yet. After 3DIV none of the cultures contained any GFAP positive astrocytes or O4 positive oligodendrocytes. Primary mesencephalic cultures contained a high proportion of TH positive neurons, presumably dopaminergic, that were rarely identified in cortical or striatal progenitor cultures.

With continuous culture in serum-free conditions, the progenitors mainly differentiated into post-mitotic neurons and for each region the majority of cells stained positive for TuJ after one week in culture. At this time point the onset of gliogenesis could be detected in striatal and mesencephalic cultures by a small number of astrocytes (Figure 3-4K) and oligodendrocytes (Figure 3-4G), a parallel development as shown *in vivo* (Williams et al., 1985). However, glial cells represented only an insignificant portion (less than 0.1%) of total cells and it was reported before that the chosen serum-free conditions do not support glial proliferation or differentiation (Walicke and Baird, 1988; Bouvier and Mytilineou, 1995; Wood et al., 2003). The increase of neurons in cortical and mesencephalic cultures is accompanied by a significant decrease of nestin positive progenitors in these cultures, reflecting the differentiation of immature neuroblasts into mature post-mitotic neurons. Although the number of total neurons in mesencephalic cultures had increased within seven days of culture, the proportion of TH positive neurons

decreased, suggesting a limited proliferation of dopamine neuroblasts and an increase in cell death of particularly dopaminergic neurons under the chosen culture conditions. TH expressing cells showed signs of neuronal death after one week in culture visible by disintegrated processes.

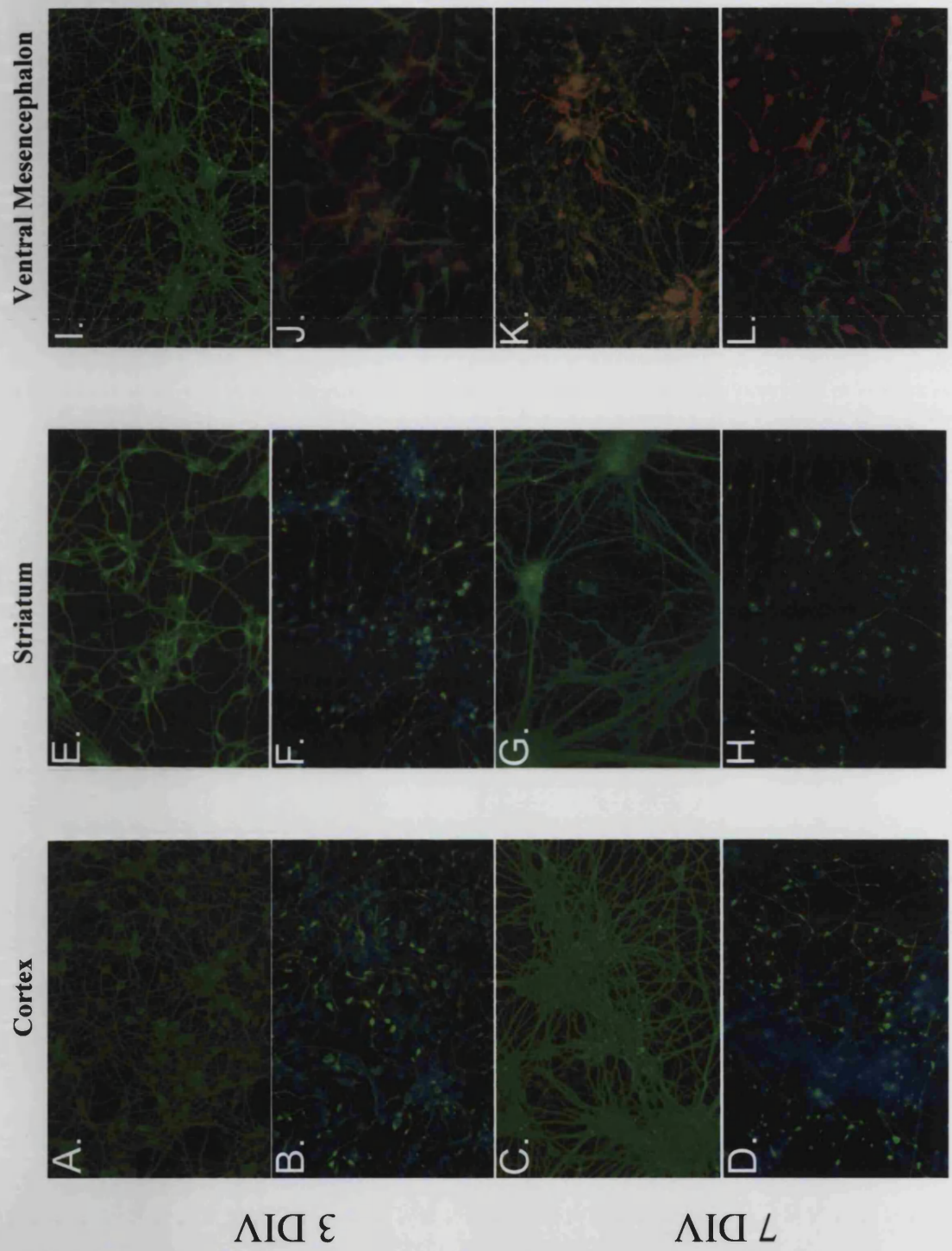


Figure 3-4. : Characterization of neural progenitor cultures. Cortex (A-D), striatum (E-H) and ventral mesencephalon (I-L) from E14 Sprague-Dawley rats were dissected, trypsin treated and triturated into a single cell suspension. Cells were plated at a density of $1x10^5$ viable cells per well on PLL/Laminin coated glass coverslips and maintained in serum free Neurobasal media supplemented with B27. On 1 DIV and 3 DIV cells were fixed with 4%PFA. Cells were either (A, E, I and C, G, K) triple-stained for the neuronal marker TuJ1 (Alexa 488: green), the astrocytic marker GFAP (Alexa 546: red) and the oligodendroglial marker O4 (Alexa 350: blue), or (B, F, J and D, H, L) stained for the neurofilament marker nestin (FITC: green), the dopaminergic marker TH (Alexa 546: red) and DAPI (blue). 40x magnification.

3.3.1.2 Gene delivery to neural progenitors depends on the viral backbone

Primary neurons and neural progenitor cells derived from cortex, striatum and ventral mesencephalon were infected on 1DIV at increasing multiplicities of infection (m.o.i.), either with the highly disabled construct 1764/27-/4-/pR19hGFP or with the less disabled RL1+/27+/4-/pR19hGFPwpre. In order to determine transduction efficiency, cells were fixed three days after infection and GFP expression was visualised under an epifluorescent microscope (Figure 3-5 and Figure 3-6). Independent of the viral backbone, transduction efficiency increased, as expected, with higher multiplicity of infection. Recombinant gene expression significantly depended on the chosen viral backbone and the neurogenic region the progenitor cells were generated from.

Highly disabled virus only gave a reasonable gene delivery to mesencephalic neural progenitors when infected with a high viral dose (m.o.i. of 10). However, even at such high multiplicities cortical or striatal progenitor cells were only rarely transduced with 1764/27-/4-/pR19hGFP. Infected mesencephalic cells showed different levels in the intensity of GFP expression. This suggests that single cells were infected with multiple virions resulting in stronger transgene expression, or alternatively, that cell type dependent differences affected transgene expression from the pR19 promoter cassette. GFP expressing cells did not show any signs of cytotoxicity due to viral infection compared to uninfected control cultures. Reporter gene expressing cells had large, flat cell bodies with long processes. Even at an m.o.i. of 10, only an insignificant number of transduced cells showed cytopathic effects, evident as rounded cells with retracted or without processes. GFP expression was localised in the cell body as well as in the processes of the progenitor cells. Infections of mesencephalic cultures at m.o.i.'s higher than 10, did not result in a proportional increase of transduction efficiency, suggesting that cells not expressing the transgene are either not susceptible to viral infection with this construct or expression from the promoter cassette is not supported in these cells.

A significantly higher gene delivery to primary neural progenitor cells was achieved with the less disabled construct RL1+/27+/4-/pR19hGFPwpre (Figure 3-6). While low viral amounts (m.o.i. of 1) resulted in transduction of only a small number of progenitors, a high number of cells were expressing the reporter gene after infections at an m.o.i. of 5 and 10, independently of the neurogenic region the cells were isolated from. Reporter gene expression was observed as soon as one day post transduction and the expression levels were highest three days after infection. Although GFP expression was localised in the cell bodies and processes for the majority of progenitor cells, an increased number of rounded cells without or retracted processes were evident at high virus doses. As discussed in more detail later in this chapter, toxicity appeared to depend on the levels and the overall time of reporter gene expression in culture. Cytotoxic effects caused increased cell loss and became evident three days after transduction.

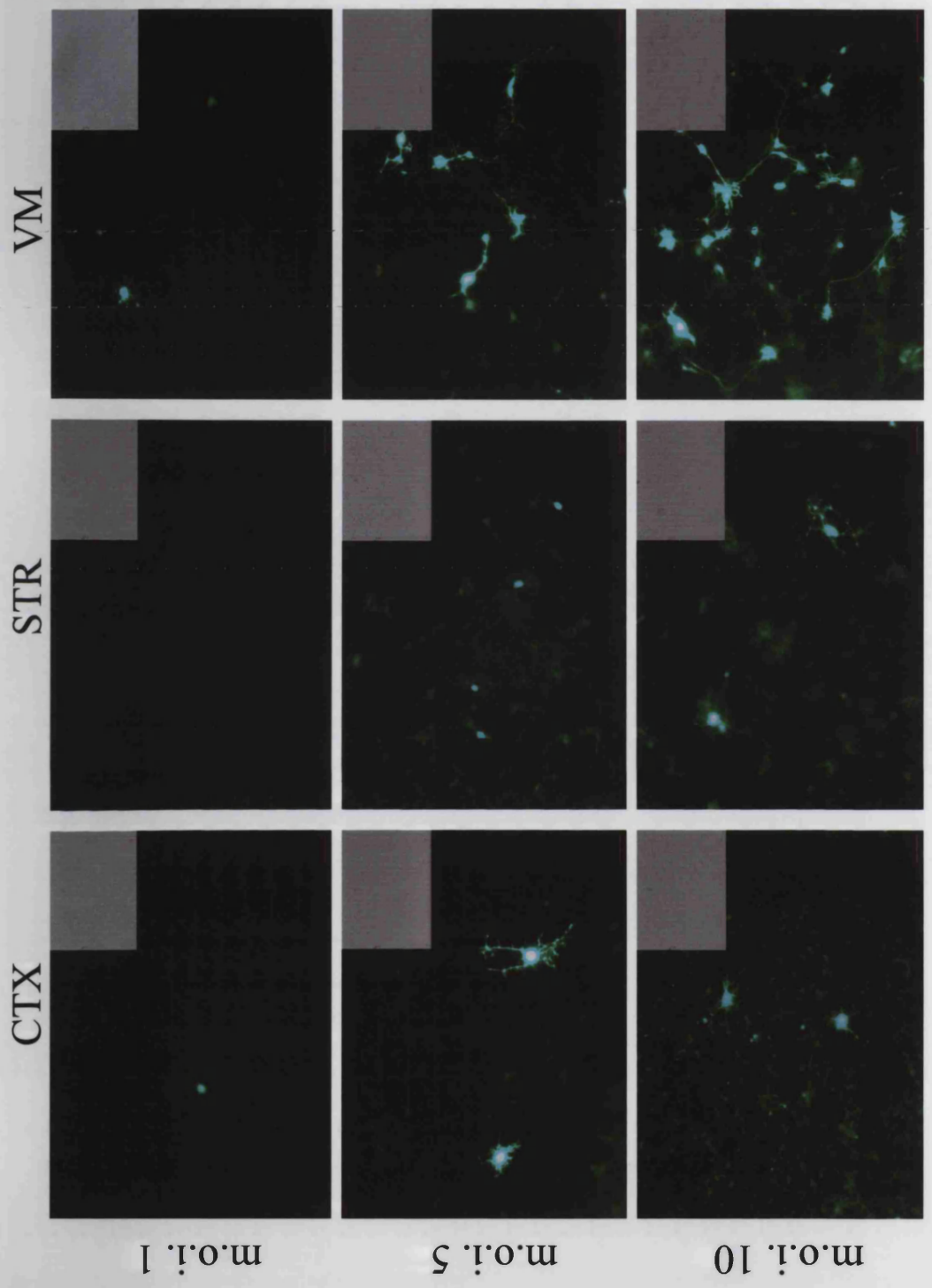


Figure 3-5: Gene delivery to neural progenitor cells using highly disabled vector 1764/27-/4-/pR19hGFP. E14 neural progenitor cells derived from cortex (CTX), striatum (STR) or ventral mesencephalon (VM) were plated at a cell density of $1x10^5$cells/well on PLL/Laminin coated cover slips. On 1 DIV cells were infected with 1764/27-/4-/pR19hGFP at an m.o.i. of 1, 5 or 10 and fixed on 4DIV with 4%PFA. GFP expressing cells were visualized under a fluorescent microscope. Small insets are pictures taken with phase contrast. Scale bar represents 50µm.

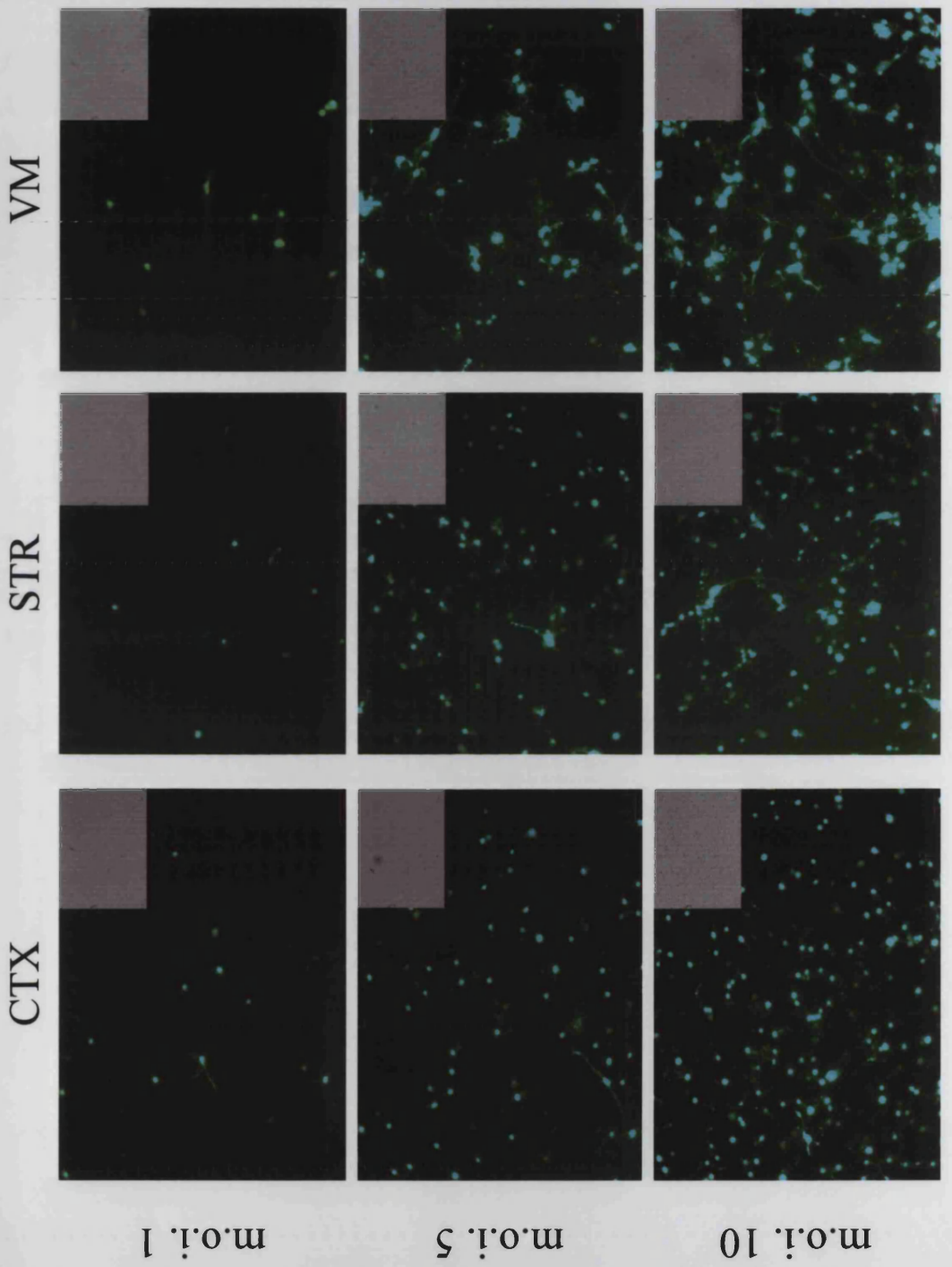


Figure 3-6: Gene delivery to neural progenitor cells using less disabled vector RL1+/27+/4-/pR19hGFPwpre. E14 neural progenitor cells derived from cortex (CTX), striatum (STR) or ventral mesencephalon (VM) were plated at a cell density of $1x10^5$cells/well on PLL/Laminin coated cover slips. On 1 DIV cells were infected with RL1+/27+/4-/pR19hGFPwpre at an m.o.i. of 1, 5 or 10 and fixed on 4DIV with 4%PFA. GFP expressing cells were visualized under a fluorescent microscope. Small insets are pictures taken with phase contrast. Scale bar represents 50µm.

Gene delivery to cortical, striatal and mesencephalic derived neurons and neural progenitor cells was quantified for infections with RL1+/27+/4-/pR19hGFPwpre at an m.o.i. of 10 three days post-transduction (Figure 3-7). Although, the highest gene delivery was found in mesencephalic derived progenitor cells compared to cortical or striatal progenitor cells, the differences between the three neurogenic regions are statistically not significant ($p>0.05$ in One-way ANOVA, $n=3$ experiments). Under the chosen experimental conditions, the transduction efficiency to primary neural progenitor cells varied between 14% in cortical and 30% in mesencephalic cultures. Cell loss due to cytopathic effects within the first three days post-transduction contributed to the low gene delivery rate, as well as weak reporter gene expressing cells that were difficult to detect. Therefore, the calculated gene delivery efficiency only gives a rough approximation of the actual amount of transduced cells. As a quantification of reporter gene expressing cells does not reflect the levels of gene expression, the less disabled RL1+/27-/4-pR19wpre viral backbone may still give sufficient transgene expression to study e.g. biological functions of genes encoding for secreted proteins.

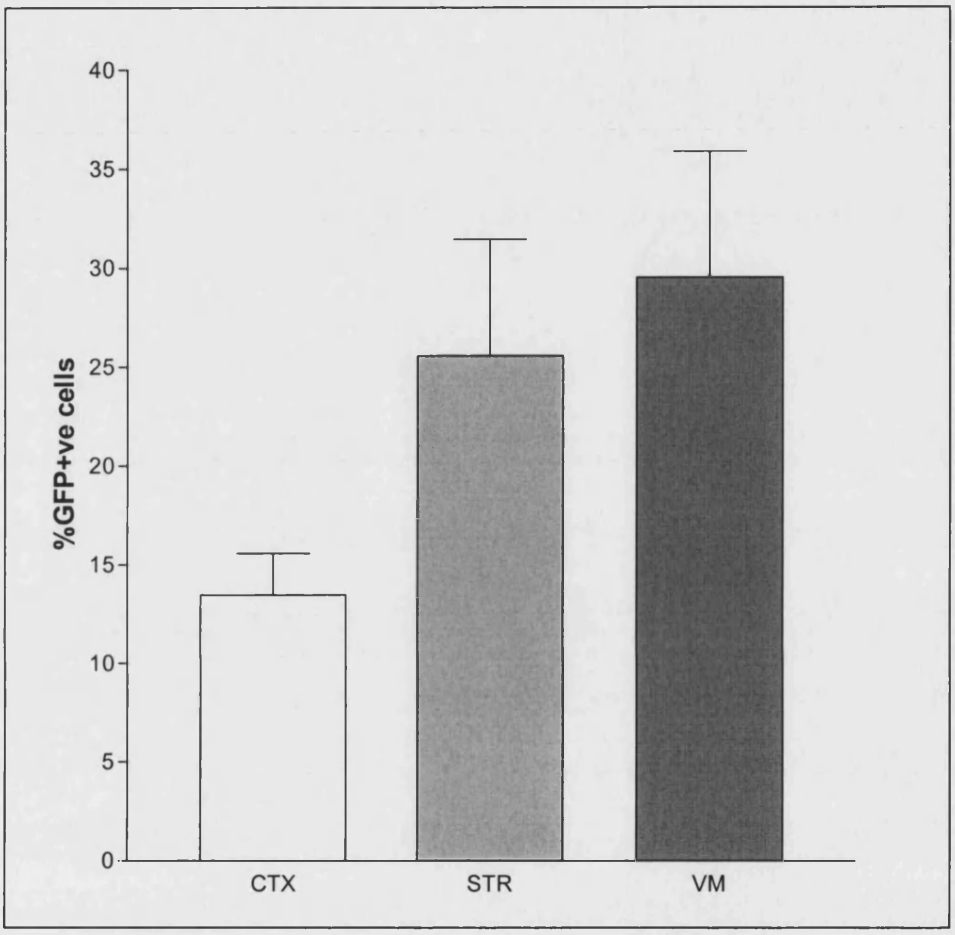


Figure 3-7: Quantification of gene delivery to cortical, striatal and mesencephalic derived neural progenitor cells. E14 primary neural progenitor cells derived from the different neurogenic regions were infected at an m.o.i. of 10 on 1DIV with RL1+/27+/4-/pR19hGFPwpre and fixed on 4DIV. The percentage of GFP expressing cells was determined from 10 different fields in duplicates under a fluorescent microscope. Data presented are the means of GFP positive cells as percentage of total cell number with SEM from three independent experiments.

3.3.1.3 Gene delivery depends on the age of the neuronal culture

As shown previously in this chapter, neural progenitor cells follow maturation *in vitro* noticeable by a change of cell morphology and cell fate towards a distinct post-mitotic neuronal character. The age of the neuronal culture may also affect gene delivery efficiency. Ventral mesencephalic progenitor cells were infected on 3 DIV and 5 DIV with either highly disabled 1764/27-/4-/pR19hGFP or less disabled RL1+/27+/4-pR19hGFPwpre. Reporter gene expression was monitored under the fluorescent microscope three days post-transduction (Figure 3-8). Gene delivery efficiency increased for both viral constructs in a time dependent course. Infection of neural progenitor cells at later culture times, when they had matured into more post-mitotic neurons, resulted in higher gene delivery efficiencies compared to earlier time points. Transduction of mesencephalic progenitors with RL1+/27+/4-pR19hGFPwpre that had been cultured for five days prior to infection, resulted in almost 100% transduction efficiency. Even with the highly disabled viral backbone 1764/27-/4-/pR19hGFP, that only gives a poor gene delivery rate after infection of immature progenitor cultures, a reasonable gene delivery was achieved when more mature neural progenitor cultures were infected. Strikingly, viral transduction at later culture time points also resulted in far less cytotoxic effects. The majority of infected cells showed healthy cell morphology with GFP expression in both soma and processes. The neurite processes were not disintegrated and far less cells were rounded up as observed after viral infection on 1 DIV. This result suggests that gene delivery efficiency not only depends on the dose of virus applied, but to a high degree to the maturation stage of the neural progenitor culture.

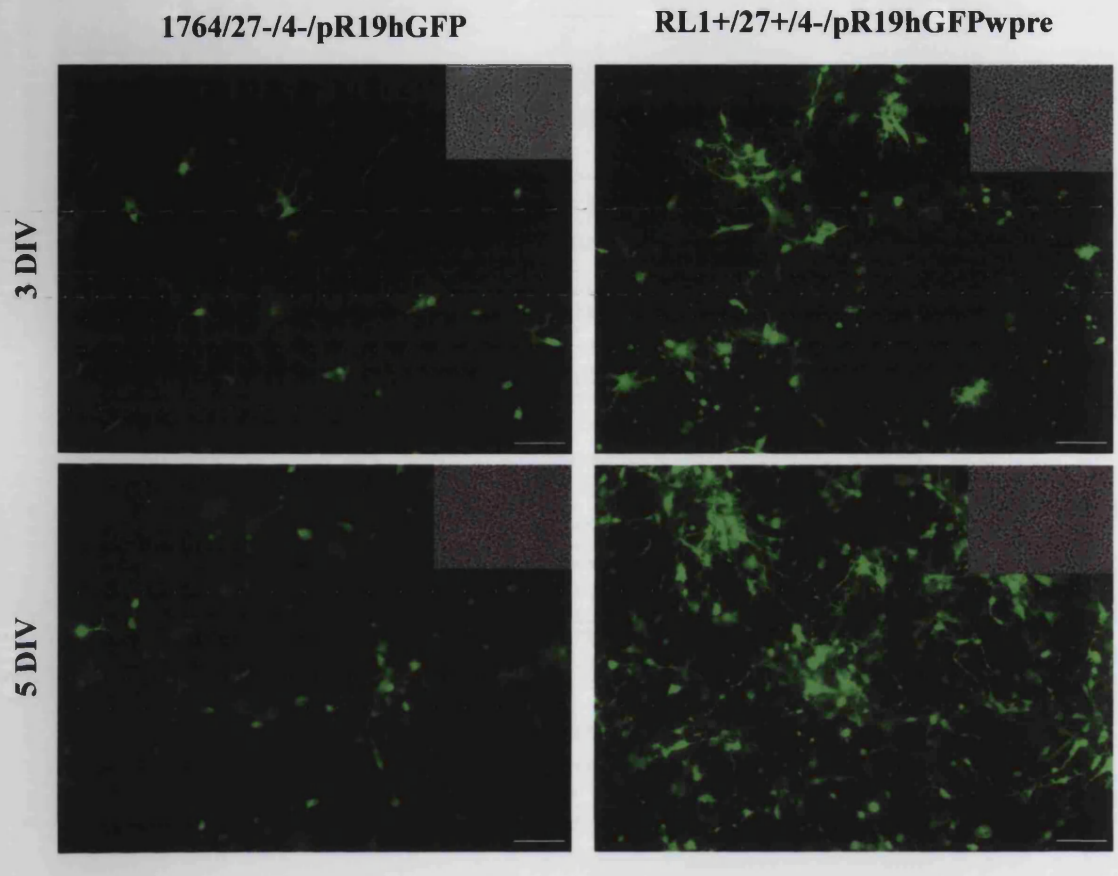


Figure 3-8: Gene delivery efficiency depends on the age of the neural progenitor culture. E14 primary ventral mesencephalic cells were plated at a cell density of $1x10^5$ cells per well on PLL/Laminin coated glass cover slips. Cells were infected at an m.o.i. of 5 with highly disabled 1764/27-/4-/pR19hGFP or less disabled RL1+/27+/4-/pR19hGFPwpre on 3 DIV and 5 DIV, respectively. Three days post-transduction cells were fixed with 4%PFA and reporter gene expression monitored under a fluorescent microscope. Insets are pictures taken with phase contrast. Scale bar represents 50µm.

3.3.1.4 Phenotypic identity of the infected neural progenitor cell

In order to identify the antigenic character of the transduced neural progenitor cells, immunocytochemical staining was performed for neuronal, glial and neurofilamental markers. Neural progenitor cells from the three distinct neurogenic regions were infected with RL1+/27+/4-pR19hGFPwpre on 1 DIV and fixed three days post-transduction. The transduced progenitor cells were stained for TuJ1 as neuronal marker, GFAP as marker for astrocytes, nestin as marker for undifferentiated neuroepithelia and TH as marker for midbrain dopaminergic neurons (Figure 3-9).

In accordance with the previously described characterization of neural progenitors, only a very few astrocytes staining for GFAP were found in the cultures. The majority of transduced cells were of neuronal identity as demonstrated by GFP/TuJ1 double labelled cells. Despite of the growth factor free culture condition transduced progenitor cells were found that stained positive for nestin. Hence, some of the infected progenitors remained in an undifferentiated fate.

Progenitor cells derived from the ventral mesencephalon contained only a low number of TH/GFP double labelled neurons. Although a high number of GFP/TuJ double labelled cells were detected in these cultures, only less than approximately 1% of the total TH positive cells expressed the reporter gene. As the majority of dopaminergic neurons differentiated into mature dopaminergic neurons or neurons with a restricted dopamine fate prior to cell preparation *in vivo*, this may reflect a rather low susceptibility of post-mitotic primary dopaminergic neurons to viral transduction with HSV-1. As a high proportion of nestin positive cells were found in progenitor cultures on 1 DIV, the GFP/TuJ1 double labelled cells may have resulted from infection of these undifferentiated neuroblasts that fully differentiated into post-mitotic neurons after viral transduction. As cultures were prepared from E14 embryos, differentiation of dopaminergic neurons had already been completed and the cultures contained under the chosen growth factor free conditions only a low number of dopamine neuroblasts available to viral transduction.

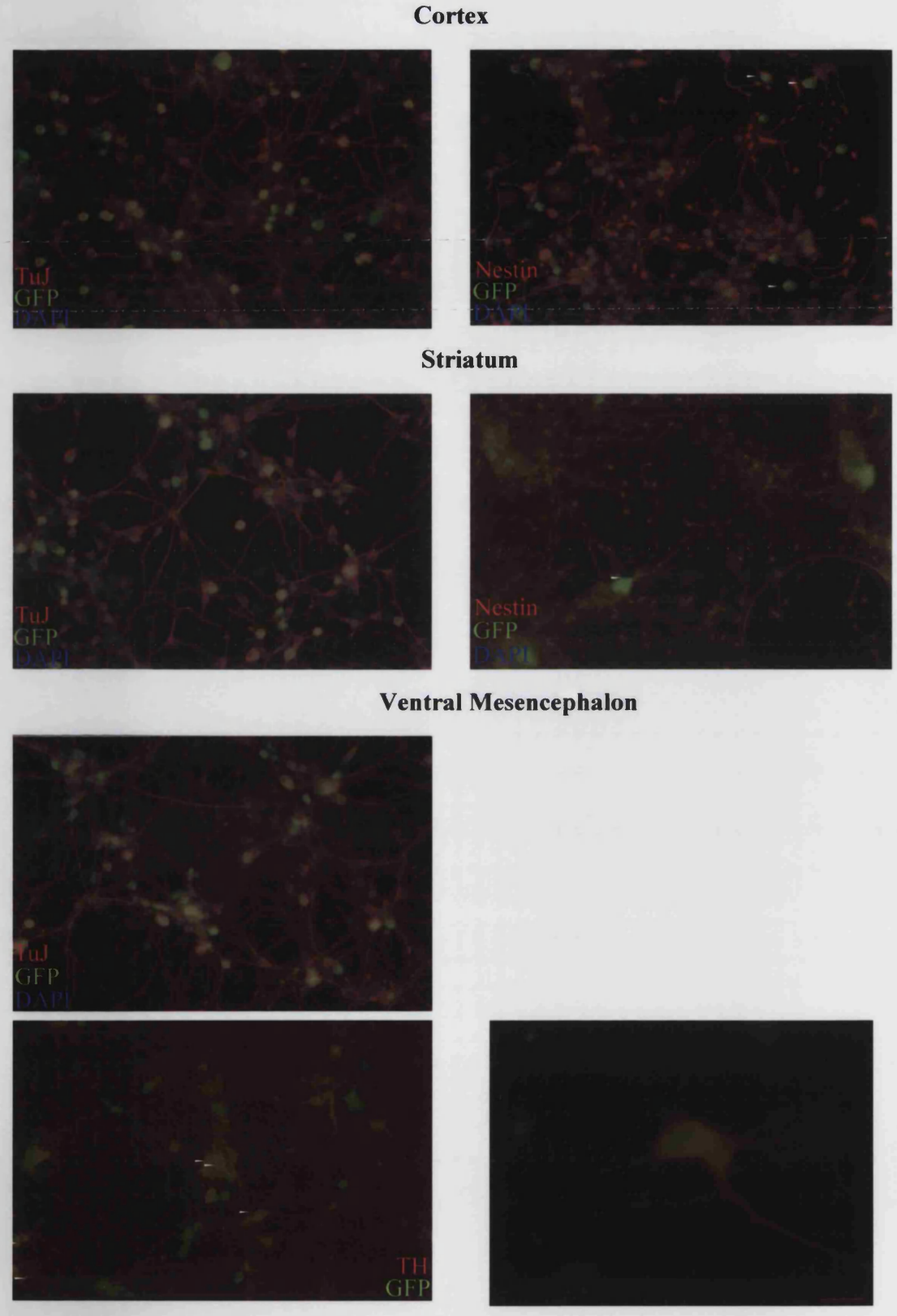


Figure 3-9: Characterization of transduced neural progenitor cells. Primary neural cells derived from E14 cortex, striatum and ventral mesencephalon were infected on 1DIV with RL1+/27+/4-pR19hGFP at an m.o.i. of 10. On 4 DIV cells were fixed with 4%PFA and stained for the neuronal marker TuJ and the neurofilament marker nestin. Mesencephalic progenitors were also stained for tyrosine hydroxylase (TH). Scale bar represents 10 µm.

3.3.1.5 Effects of viral transduction on neurogenesis and the number of dopaminergic neurons

Vector cytotoxicity was studied for the less disabled virus RL1+/27+/4- in primary neural progenitor culture derived from cortex, striatum and ventral mesencephalon. Cells infected with RL1+/27+/4-/pR19hGFP at an m.o.i. of 1, 5 and 10 were fixed three days after infection and stained for the neuronal marker TuJ1 (Figure 3-10). Cytotoxic effects were examined by changes of cell morphology, noticeable by lost or disintegrated cells processes, resulting in rounded, often enlarged somas that easily detach from the cell culture surface and float in the cell culture supernatant. One day after infection GFP expression could be visualised in processes and cell bodies of transduced neuronal cells. At this time point cytotoxic effects were not apparent, the cells showed healthy morphology and no cells were floating in the supernatant.

With continuous culture and reporter gene expression the viability of transduced neural progenitor cells decreased on 3 and 4 DIV. An increased number of GFP expressing cells that co-labelled with the neuronal marker TuJ1 lost or retracted their neuronal processes. These cytotoxic effects were observed in cortical, striatal and mesencephalic derived neuronal cultures and increased with the viral dose added. However, control cultures that were incubated with serum free media hardly contained any neurons with an altered cell morphology which confirms that viral transduction and not the culture conditions caused the observed cytotoxic effects. Strikingly, GFP fluorescence was stronger in cells showing cytotoxic effects than in transduced cells with unaltered neuronal morphology. It cannot be concluded from these data, if the intense fluorescence is the result of increased reporter gene expression or the rounded cell shape compared to a polygonal cell shape in healthy neurons. However, Detrait et al. showed that toxicity of a given reporter gene product correlated with the level of expression of the gene (Detrait et al., 2002) suggesting that high level reporter gene expression contributed to the observed cytopathic effects. This is further confirmed by the observation that cytotoxic effects are time-dependent and increase with continuous GFP expression in the transduced neurons resulting in an accumulation of potentially toxic reporter gene product.

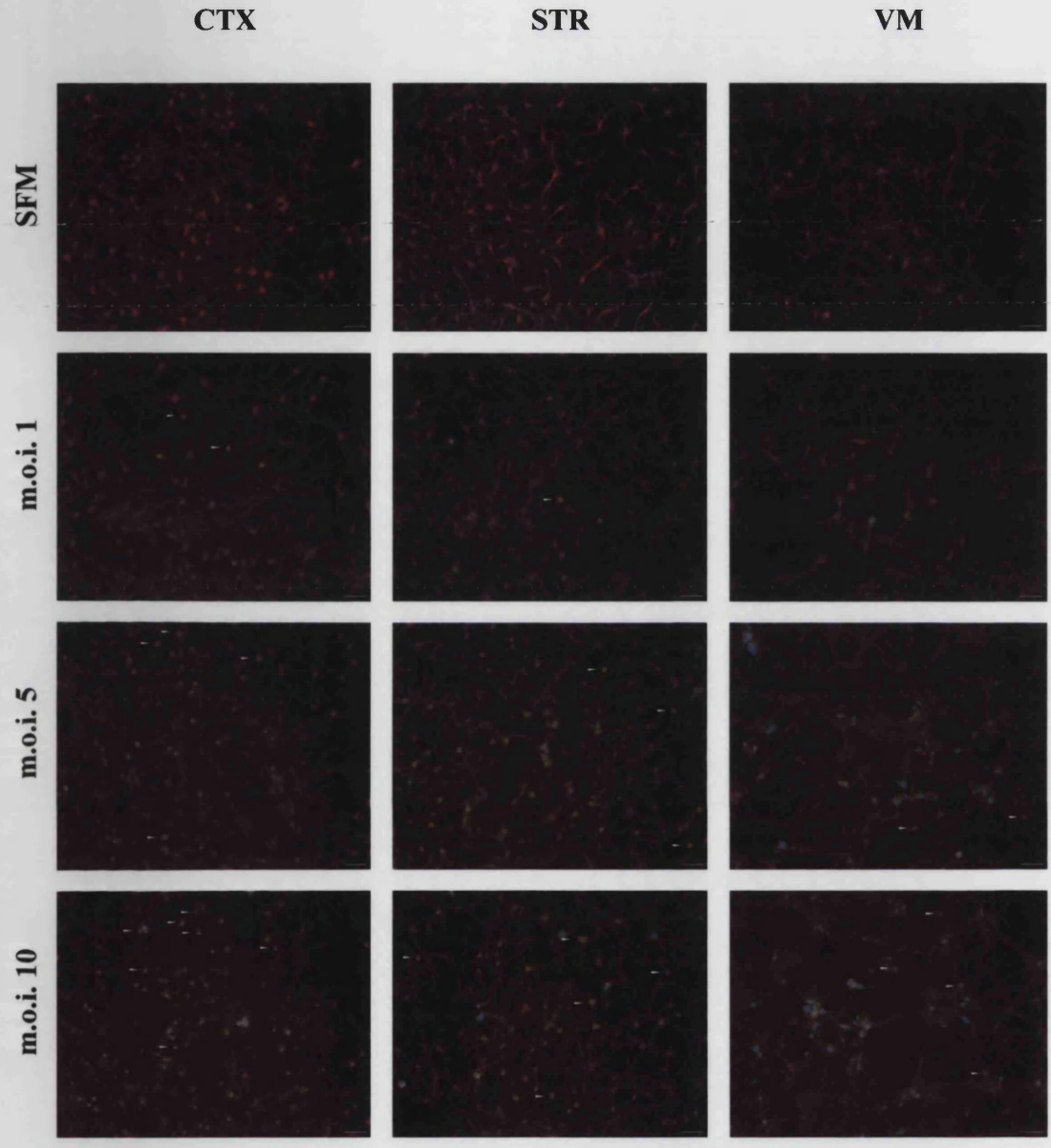


Figure 3-10: Effects of viral transduction on neurogenesis. Primary neural cultures were prepared from E14 cortex (CTX), striatum (STR) and ventral mesencephalon (VM). Cells were plated at a cell density of $1x10^5$cells/well on PLL/laminin coated coverslips and infected on 1DIV with RL1+/27+/4-pR10hGFPwpre at the shown multiplicity of infection or with serum-free media (SFM). On 4DIV cells were fixed with 4% PFA and stained for the neuronal marker TuJ1 (Alexa 546, red). GFP expression was visualized under the fluorescent microscope. Cytotoxic effects due to viral infection and/or reporter gene expression were observed with increasing viral amounts independent of the neurogenic region. Transduced neurons showing cytopathic effects round up and have disintegrated or no processes (arrow heads). Scale bar represents 50µm.

To quantify the observed cytotoxic effects and the impact of viral transduction on neurogenesis in primary neural cultures, the total amount of TuJ1 positive neurons and the proportion of neurons with cytopathic effects were determined (Table 3-2). Viral transduction with RL1+27+/4-pR19hGFPwpre at high m.o.i.'s significantly reduced the total number of TuJ1 positive neurons in cortical and mesencephalic cultures. The neuronal cell loss correlated with a significant increase in the number of neurons showing cytotoxic effects. While only about 1% of the neurons were rounded in control cultures, about 14% (striatum) to 20% (mesencephalon) of the neurons showed cytopathic effects in virally transduced progenitor cultures. About 40% of the TuJ1 positive neurons were transduced as demonstrated by GFP expression for mesencephalic derived cultures at the chosen multiplicity. These data confirmed that although RL1+27+/4-pR19hGFPwpre efficiently infected mesencephalic neurons and progenitor cells, gene delivery is accompanied by a decreased neuronal viability and profound neuronal cell loss in these cultures.

Cortex

	Total TuJ1 (%)	rounded TuJ1 (%)
SFM	58.38±1.57	1.30±0.60
RL1+hGFPwpre	36.36±2.26	16.09±3.12

Striatum

	Total TuJ1 (%)	rounded TuJ1 (%)
SFM	61.29±1.04	1.02±0.11
RL1+hGFPwpre	51.44±2.56	14.02±2.99

Ventral Mesencephalon

	Total TuJ1 (%)	rounded TuJ1 (%)	TuJ1/GFP (%)
SFM	56.53±0.12	1.44±0.82	n/a
RL1+hGFPwpre	43.38±1.99	20.42±7.64	41.6±2.59

Table 3-2: Neuronal loss due to viral transduction. E14 primary neural cultures from cortex, striatum and ventral mesencephalon were infected on 1DIV with RL1+/27+/4-pR19hGFPwpre at an m.o.i. of 10 and fixed on 4DIV. Cells were stained for the neuronal marker TuJ. The number of total neurons as percentage of cells and the number of cells without processes (rounded) as percentage of total neurons was counted from 10 different fields in duplicates. For mesencephalic derived neural progenitor cells the number of GFP+ve infected neurons was determined as percentage of total TuJ1+ve neurons. Data are presented as means with standard error of the means (SEM) from three independent experiments. Viral transduction causes a statistically significant loss of TuJ+ve neurons in cortical ([a] two-tailed t-test, $p<0.01$, n=3) and striatal ([b] two-tailed t-test, $p<0.02$, n=3) progenitor cultures. Viral transduction increases in all neuronal progenitor cultures the amount of neurons showing cytotoxic effects characterized by the loss of neuronal processes.

3.3.1.6 Effects of viral transduction on the number of dopaminergic neurons

As characterized earlier in this chapter only a proportion of the total neurons in mesencephalic cultures are dopaminergic, identified here by the expression of tyrosine hydroxylase. With the aim to use HSV-1 as vector for dopaminergic neurons, the effects of viral transuction on the number of TH expressing neurons was determined (Figure 3-11). Primary mesencephalic progenitor cells were infected with RL1+27+/4-pR19hGFPwpre at increasing multiplicities and the number of TH expressing cells was assessed three days post-transduction. Viral transduction with RL1+27+/4-pR19hGFPwpre does not appear to affect the number of dopaminergic neurons compared to control cultures. Even at high multiplicities no significant loss of dopaminergic neurons was observed. Similar results were also found after infection of mesencephalic neuronal progenitor cultures with the highly disabled 1764/27-/4-pR19hGFP after infection on 3 DIV (Figure 3-11). This result seems to be contrary to the profound neuronal loss in mesencephalic progenitors as described above. However, as shown earlier in this chapter RL1+27+/4-pR19hGFPwpre preferentially transduced immature neural progenitor cells that differentiate into post-mitotic TuJ1 positive neurons, but not necessarily dopaminergic neurons. Although some dopaminergic neurons were demonstrated to be infected by co-labelling of TH and GFP (Figure 3-9), the majority of dopaminergic neurons were not expressing the reporter gene suggesting a limited gene delivery to post-mitotic dopaminergic neurons. This lack of gene delivery to dopaminergic neurons explains the unaltered number of dopaminergic neurons in mesencephalic cultures. It also suggests that viral transduction and possibly expression of residual viral genes or reporter genes induced a cytopathic cascade in transduced neurons, but not contaminating components of the viral stock, such as non-infectious viral particles or cell debris from the producer cell line.

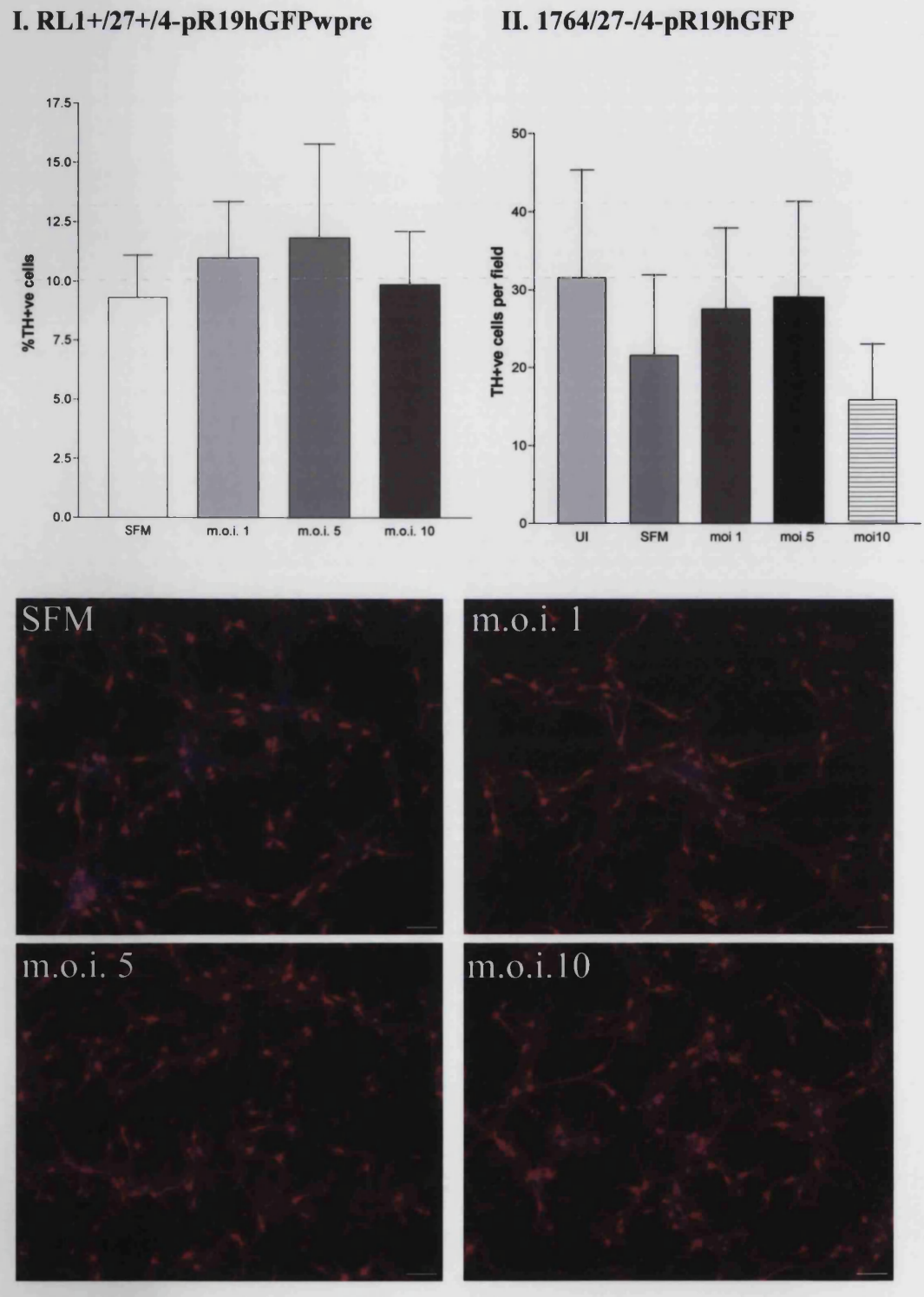


Figure 3-11: Effects of viral transduction on the amount of dopaminergic neurons. Graph illustrates no significant loss of dopaminergic neurons due to viral transduction. E14 mesencephalic neural precursor cells were infected on 1 DIV with RL1+/27+/4-/pR19hGFP or 1764/27-/4-pR19hGFP at the shown m.o.i. and fixed on 4DIV. Dopaminergic neurons were characterized by immunocytochemical staining for tyrosine hydroxylase (Alexa 546, red) and the cell nuclei were treated with DAPI (blue). Data represent the means of TH+ve cells as percentage of total cell number with SEM from three independent experiments. Scale bar represents 50µm.

3.3.2 HSV-1 as a gene delivery vector to neurospheres as model for neural stem cells

Neural stem cells (NSCs) isolated from the developing (Reynolds et al., 1992; Uchida et al., 2000) or adult (Reynolds and Weiss, 1992; Gritti et al., 1996; Palmer et al., 1997; Palmer et al., 1999) CNS can be expanded as spherical aggregates, or 'neurospheres', to a large number of cells that retain their multipotent neural characteristics (Reynolds et al., 1992; Vescovi et al., 1993; Gritti et al., 1996) and thus, are available for *ex vivo* gene transfer manipulations. Here it is reported for the first time that replication-deficient HSV-1 is also a promising gene delivery vector to rodent and human neurospheres as it efficiently transduces NSCs without causing major cytotoxic insults or interfering with the cellular metabolism of the NSC.

3.3.2.1 The effects of heparin on viral transduction

A prerequisite for viral transduction is that the target cells express the necessary cellular receptors for HSV-1 attachment. The proteoglycan heparin sulphate and the mitogen FGF2 are of significant importance in neurodevelopment (for review see Yamaguchi, 2001) and their interaction also plays a crucial role in the division and differentiation of neuronal precursor cells from the embryonic and adult CNS (for reviews see Vaccarino et al., 1999; Ford-Perriss et al., 2001). The evidence that heparin sulphate has been identified as a major factor in binding of HSV to the cell surface (WuDunn and Spear, 1989) and reports indicating that the basic fibroblast growth factor receptor itself plays a role in viral transduction (Kaner et al., 1990; Muggeridge et al., 1992) prompted us to the hypothesis that HSV-1 may be a potent candidate for efficient gene delivery to neural stem cells.

Further, heparin plays an important role for the stability and activity of FGF2 (Gospodarowicz and Cheng, 1986; Saksela et al., 1988) and particularly potentiates the mitogenic effects of FGF2 in mesencephalic neurospheres (^{mes}NS) (Caldwell and Svendsen, 1998). ^{mes}NS grow significantly slower than rodent neurospheres generated from cortex (^{ctx}NS) or striatum (^{str}NS) (Ostenfeld et al., 2002) and thus, their

expansion to a high cell number required for experiments is limited in the absence of heparin. Therefore, neurospheres are routinely expanded in the presence of heparin at a final concentration of 5μg/ml in the culture medium when FGF2 is used as mitogen. Although it has been shown by Immergluck et al. that the heparin concentration required to inhibit viral transduction in neuronal cells is far higher (IC_{50} ~30μg/ml) (Immergluck et al., 1998), we determined the potentially inhibiting effects of heparin on viral transduction of mesencephalic neurospheres.

^{mes}NS expanded in FGF2 (20ng/ml) containing culture media in the presence or absence of heparin (5μg/ml) were infected with highly disabled 1764/27-/4-/pR19hGFP either as whole neurosphere or after dissociation of neurospheres into single cells plated on PLL/Laminin coated cover slips. To minimize further cell division of transduced NSC, the infected cells were maintained for another two days in media without FGF2. GFP expression was visualized under a fluorescent microscope and could first be detected as soon as one day post-transduction, demonstrating that ^{mes}NS generally were susceptible to viral transduction with the highly disabled construct. Growth factor removal induces differentiation of expanded NSC (Gage, 2000) and transduced NSC within the neurosphere appear to extend long, branched processes (Figure 3-12).

The presence of heparin during the expansion phase of the ^{mes}NS significantly affected viral transduction. Heparin expanded cultures showed reduced gene delivery after infection of whole neurospheres or more prominently after transduction of dissociated ^{mes}NSCs. At the lower multiplicity chosen (m.o.i 5) hardly any reporter gene expressing NSCs were detected in dissociated cultures and only a low gene delivery was observed in whole neurospheres when heparin was present prior to infection. However, in the absence of heparin the majority of cells in dissociated and neurospheres cultures were expressing GFP at the same multiplicity. The inhibiting effects of heparin on viral transduction could be slightly overcome by increasing the multiplicity of infection. Heparin expanded ^{mes}NS infected at higher multiplicities (m.o.i. 10) contained more reporter gene expressing cells as is particularly evident for infections of dissociated cultures. However, doubling the viral amount did not

accomplish the gene delivery efficiency observed for cultures expanded in the absence of the proteoglycan but infected at the lower multiplicity.

The inhibiting effects of heparin were not significantly decreased by rinsing the neurospheres (3x) in heparin-free media prior to viral infection. This suggests that residual heparin associated to the cells prevents viral attachment to the occupied receptors or alternatively, binds to the virion and thus, occupies virion sites necessary for attachment to cell surface heparan sulphates. This observation confirms previous studies showing that heparin inhibits attachment only when present during virion adsorption in endothelial cells (WuDunn and Spear, 1989), as well as in primary neuronal cultures (Immergluck et al., 1998).

Consequently, NSCs cannot be expanded in the presence of heparin in this study when viral transduction is anticipated. The significantly reduced proliferation rates of ^{mes}NS in the absence of heparin make clonal analysis of transduced ^{mes}NSC difficult for these studies. Therefore, ^{mes}NS cultures for gene delivery studies were seeded at non-clonal densities (100 cells/μl vs. 20 cells/μl in clonal density experiments) in proliferation media without heparin. As cortical and striatal NSC have higher proliferation rates even in the absence of heparin, they were seeded at clonal density (20 viable cells/μl) in heparin-free expansion medium for all further studies described. It has been demonstrated previously by mixing marked and unmarked cells that culturing cells at this density will result in clonal neurosphere colonies, as form in single-cell cultures, and that neurospheres do not arise as a result of cell aggregation at the cell culture densities used here (Morshead et al., 1998; Tropepe et al., 2000).

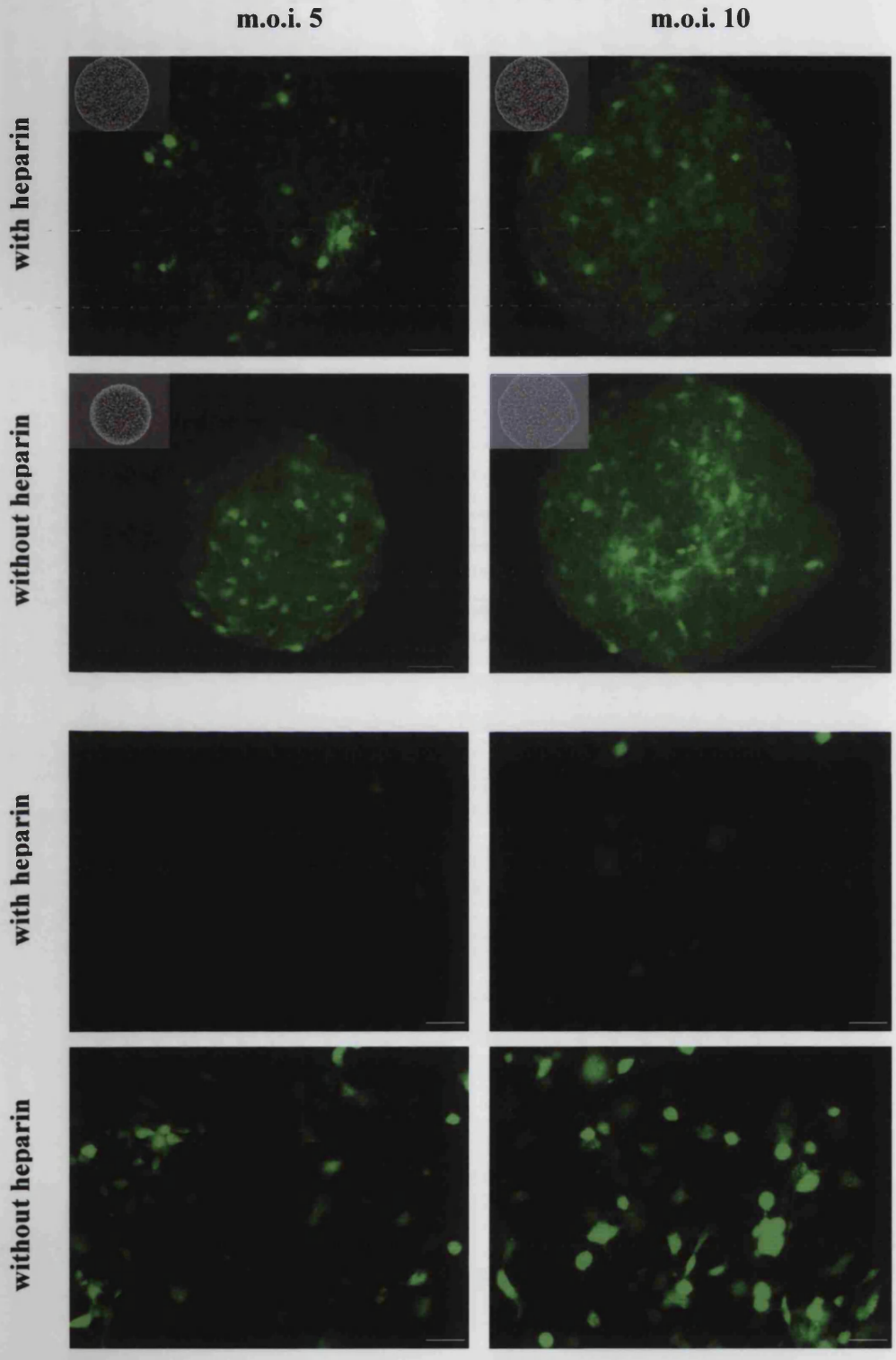


Figure 3-12: Effects of heparin on viral transduction of mesencephalic neurospheres. Neural precursor cells derived from E14 ventral mesencephalon were expanded as neurosphere cultures in media containing FGF2 (20ng/ml) either in the presence or absence of heparin (5µg/ml). Either the whole neurospheres (top pictures) or dissociated cultures (bottom pictures) generated from mesencephalic NSC were infected at an m.o.i. of 5 and 10. Cells were maintained for another two days in the absence of growth factors and heparin, then fixed with 4% PFA and reporter gene expression visualised under a fluorescent microscope. Scale bars represent 100µm in top pictures and 50µm in bottom pictures.

3.3.2.2 Highly disabled HSV-1 efficiently transduces mesencephalic neural stem cells

To determine the approximate gene delivery rate to mesencephalic neurospheres, ^{mes}NS were infected at increasing multiplicities of 1, 10 and 30 with highly disabled 1764/27-/4-pR19hGFP. In order to calculate the required viral amount for infection, the cell number of the neurospheres was assessed by selecting 100 medium sized neurospheres (about 300μm diameter) for each treatment. The neurospheres of one representative group were dissociated into a single cell suspension and the number of viable cells determined by trypan blue exclusion method. The selected neurospheres contained about $1x10^4$ cells per sphere. Viral infection of the whole neurospheres was performed in a small volume (50μl) of serum-free media for one hour at 37°C. Following transduction the virus containing media was removed and the neurospheres were maintained for another two days in differentiation media to avoid continuous proliferation of infected cells. Three days post-transduction the neurospheres were dissociated into a single cell suspension and GFP expression was monitored under an epifluorescent microscope using a FITC filter. The number of GFP expressing cells as percentage of total cell number was determined from 10 different fields (more than 200 cells per treatment) and the final mean calculated from three independent experiments (Figure 3-13).

GFP expression appeared evenly distributed throughout the entire neurosphere. It seemed that the virus did not only infect cells in the outer layer of the sphere surface, but also diffused inside the neurosphere to infect ^{mes}NSC even deep inside the core. Penetration of the virus may dependent on the size of the sphere and be limited by increasing diameter of the sphere. With higher viral doses the number of reporter gene expressing cells and the levels of GFP expression increased (Figure 3-13D). Infections at very high multiplicities showed a much stronger fluorescent signal than at lower m.o.i.'s or infections of larger neurospheres. Most likely, this was the effect of multiple infection events in the same neural precursor cell resulting in a transduced ^{mes}NSC harbouring the genetic information of more than one virion. The transduced neurospheres did not show obvious cytotoxic effects at the chosen multiplicities. The

surface of the spheres remained smooth and only at higher multiplicities cells of the outer layer round up and detach from the cell context (Figure 3-13C).

Although penetration of the virus inside the spheres cannot be concluded from the fluorescent images due to the three dimensional nature of the sphere, dissociation of neurospheres infected at high m.o.i.'s showed that most of the ^{mes}NSC were infected. The highly disabled construct 1764/27-/4-/pR19GFP efficiently transduced ^{mes}NS even at low multiplicities (Figure 3-13). About 40% of the precursor cells were expressing the reporter gene at an m.o.i. of 1 and gene delivery efficiency could be increased to more than 80% at m.o.i.'s of 10 or higher. Therefore, an adequate gene transfer to ^{mes}NSC could be achieved with relatively low amounts of highly disabled replication deficient HSV-1. However, the gene delivery rate does not give any information about possible adverse effects of viral transduction on cellular integrity and thus must be seen in context with the following studies considering potential modifications of the stem cell characteristics.

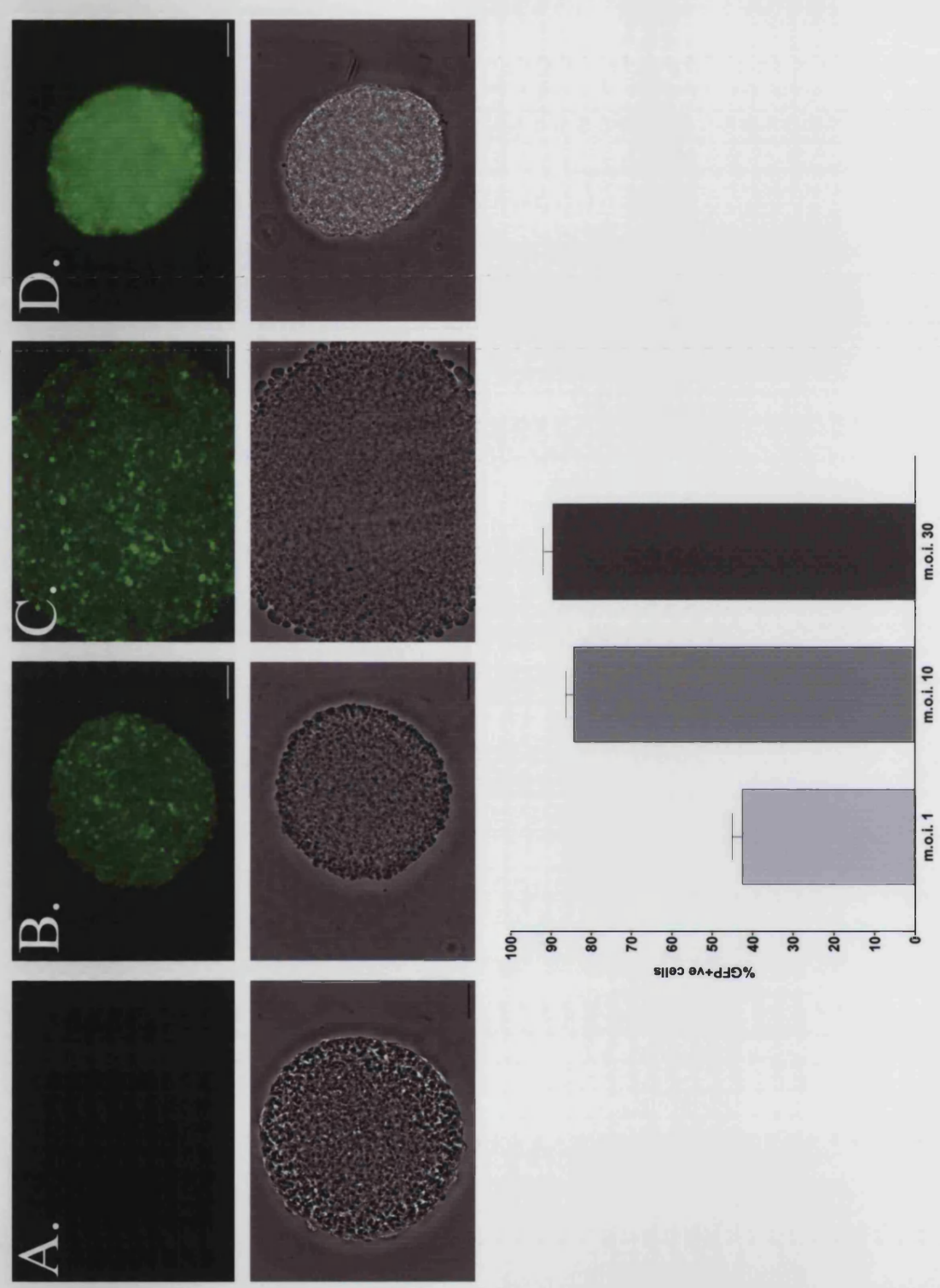


Figure 3-13: Highly disabled HSV-1 efficiently transduces mesencephalic neural precursor cells. Neurospheres derived from the ventral mesencephalon of E14 rats were expanded in FGF2 (20ng/ml) containing proliferation media in the absence of heparin. The neurospheres were infected either with SFM (A) or diluted 1764/27-/4-pR19hGFP at an m.o.i. of 1 (B), 10 (C) or 30 (D). Following infection, the media was removed and the neurospheres maintained for another two days in differentiation media. GFP expression was visualised under an epifluorescent microscope with FITC filter. The neurospheres evenly express the reporter gene. Scale bar represents 100µm, 50µm in C. As illustrated in the graph the gene delivery rate at the different m.o.i.'s was determined by dissociating the infected neurospheres into single cells. The number of GFP expressing cells presented as percentage of total cells was determined under the fluorescent microscope from 10 different fields (>200 cells per treatment). Data show the means of three independent experiments with SEM.

3.3.2.3 Gene delivery to cortical, striatal and mesencephalic neurospheres shows region specific differences

Neurospheres can be generated from various neurogenic regions of the developing CNS, including cortex, striatum and ventral mesencephalon (Svendsen and Smith, 1999). A fundamental question in this field is: do neural stem cells of distinct regions behave in a similar fashion irrespective of their origin or are they regionally specified? Several studies have addressed this question and shown by morphological and molecular analysis that neurospheres are regionally specified (Zappone et al., 2000; Ostenfeld et al., 2002; Parmar et al., 2002; Hitoshi et al., 2002). As demonstrated earlier in this chapter, primary neural progenitor cells from these distinct neurogenic regions showed differences in their susceptibility to viral transduction with disabled HSV-1. Gene delivery to striatal and mesencephalic primary neural cultures was much more efficient than to cortical neural progenitor cells. Here it was asked if transduction efficiency also depends on the region from which the neurospheres were generated.

Neurospheres were generated from E14 rat cortex (^{ctx}NS), striatum (^{str}NS) or ventral mesencephalon (^{mes}NS) and expanded for 7 days in proliferation media without heparin. Medium sized neurospheres were selected and the cell concentration determined as described before (3.3.2.2). Neurospheres of the different regions were infected at the same multiplicity (m.o.i. of 3) with three different viral constructs: the highly disabled vector 1764/27-/4-pR19hGFP and the less disabled constructs either without the wood chuck response element (RL1+/27+/4-pRhGFP) or containing the wood chuck element (RL1+/27+/4-pRhGFPwpre). Reporter gene expression was monitored three days post-transduction under an epifluorescent microscope with FITC filter (Figure 3-14).

Gene delivery efficiency depended not only on the viral vector used but also varied with the neurogenic region the neurospheres were derived from. Infections with the less disabled construct containing the wood chuck response element resulted in the highest gene expression. GFP expression was significantly reduced using the same viral backbone but without the woodchuck response element, suggesting that the

wood chuck element may have beneficial effects on transgene expression. For all three vector systems tested we found significant differences in the transduction efficiency depending on the neurogenic origin of the neurospheres. ^{ctx}NS were least susceptible to viral transduction with any of the constructs and a significant gene delivery at the chosen m.o.i. was only achieved using RL1+/27+/4-pR19hGFPwpre. All three viral constructs delivered with much higher efficiency to ^{mes}NS with the strongest GFP expressing using RL1+/27+/4-pR19hGFPwpre. Surprisingly, the less disabled construct without wood chuck response element resulted in a lower transduction efficiency than the highly disabled construct. The same effect was obvious in ^{str}NS with a significant lower reporter gene expression after infection with the less disabled vector. This is contradictory to results obtained from studies in primary neuronal progenitor cells showing significantly higher gene delivery with RL1+/27+/4-pR19hGFP compared to 1764/27-/4-pR19hGFP and maybe the result of differences in the quality of the viral high titre stocks prepared. Infection of ^{str}NS with 1764/27-/4-pR19hGFP and RL1+/27+/4-pR19hGFPwpre resulted in the strongest reporter gene expression. NSC's of the entire neurosphere were expressing GFP at very high intensity suggesting multiple transduction events in striatal neural stem cells that are highly susceptible for infection with disabled HSV-1.

This finding has consequences for the choice of viral vector to infect neurospheres generated from distinct regions. Mesencephalic and striatal derived neurospheres can be efficiently transduced with either the highly disabled or the less disabled vector. However, where cortical neurospheres are the interest of study, RL1+/27+/4-pR19hGFPwpre may be the only choice for infections at low multiplicities.

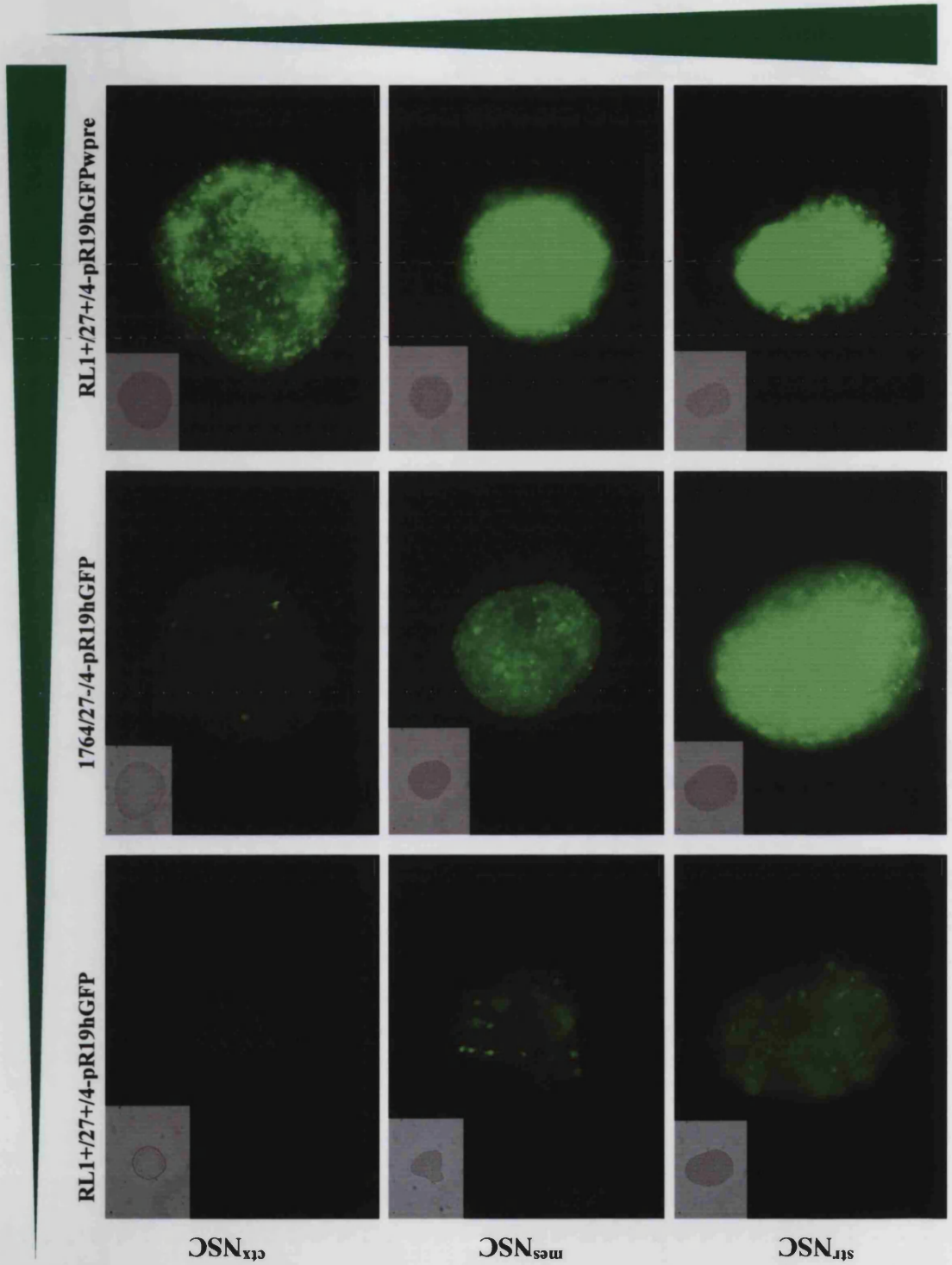


Figure 3-14: Region specific differences in the gene delivery efficiency to NSC generated from distinct neurogenic regions. Neurospheres were generated from E14 cortex (^{ctx}NSC), striatum (^{str}NSC) and ventral mesencephalon (^{mes}NSC). Selected medium sized neurospheres were infected at an m.o.i. of 5 with the highly disabled construct (1764/27-/4-pR19hGFP) or the less disabled viral vector either with the woodchuck response element (RL1+/27+/4-pR19hGFPwpre) or without (RL1+/27+/4-pR19hGFP). Reporter gene expression was monitored three days post-transduction under an epifluorescent microscope. Reporter gene expression was a function of viral construct and neurogenic region (triangle). For all constructs transduction efficiency was highest in ^{str}NSC, followed by ^{mes}NSC and lowest in ^{ctx}NSC. At the same multiplicity reporter gene expression was higher using RL1+/27+/4-pR19hGFPwpre than the highly disabled 1764/27-/4-pR19hGFP. Scale bars represent 100µm.

3.3.2.4 Transduction of mesencephalic neurospheres does not impair cell migration or proliferation

Intact neurospheres plated on poly-L-lysine/Laminin coated surfaces, attach to the substrate and form radial processes already one hour after attachment that can be characterized by radial glial marker (Caldwell and Svendsen, 1998). In time-lapse microscopy it has been shown that TuJ1-positive cells with long leading growth processes migrate along these radial fibers and onto the laminin (Caldwell and Svendsen, 1998). Individual transduced neural precursor cells are required to migrate from the plated neurosphere and onto the substrate to be analysed. Therefore, only those cells were considered that have actively migrated as progenitor cells out onto the substrate and subsequently undergone differentiation, rather than any remaining post migratory cells. This cell migration model was used to study if viral transduction inhibits formation of radial processes and migration of neuroblasts.

Mesencephalic derived neurospheres were infected with highly disabled 1764/27-/4-/pR19hGFP at increasing multiplicities. Following transduction, residual virus was removed and the neurospheres were plated on PLL/Laminin coated glass cover slips. Following transduction the ^{mes}NS were maintained in differentiation media. As soon as one hour after plating processes were visibly emerging from the neurosphere surface onto the substrate. As reporter gene expression is not apparent at this early time point, the processes may originate from transduced or untransduced ^{mes}NSC. One day after plating plenty of long processes had emerged from the neurosphere leading far distances onto the substrate. Reporter gene expression was visualised under an epifluorescent microscope showing that GFP was localised in a high number of the radial processes (Figure 3-15). Formation of processes was not obviously affected at low and medial viral amounts (m.o.i. of 1 and 10) and cells did not show any cytopathic effects. However, at high multiplicities (m.o.i. of 30) some of the plated neurospheres with strong reporter gene expression emerged significantly less processes (Figure 3-15). At this m.o.i. the processes were also shortened or disintegrated and cytopathic effects, detectable as rounded cells, became more evident. To study if neuroblasts can still evolve from the ^{mes}NSC and migrate along the radial processes, differentiated cultures were stained for the neuronal marker

TuJ1. Several TuJ1-positive neurons were found far away from the neurosphere and must have resulted from migrating neuroblasts. As shown in Figure 3-15D transduced neuroblasts retain their ability to migrate along the processes of potentially radial glial cells. Thus, the cell migration assay showed that viral transduction at low and medial multiplicities did not affect the ability to form radial processes that build the scaffold for neuroblasts to migrate along at.

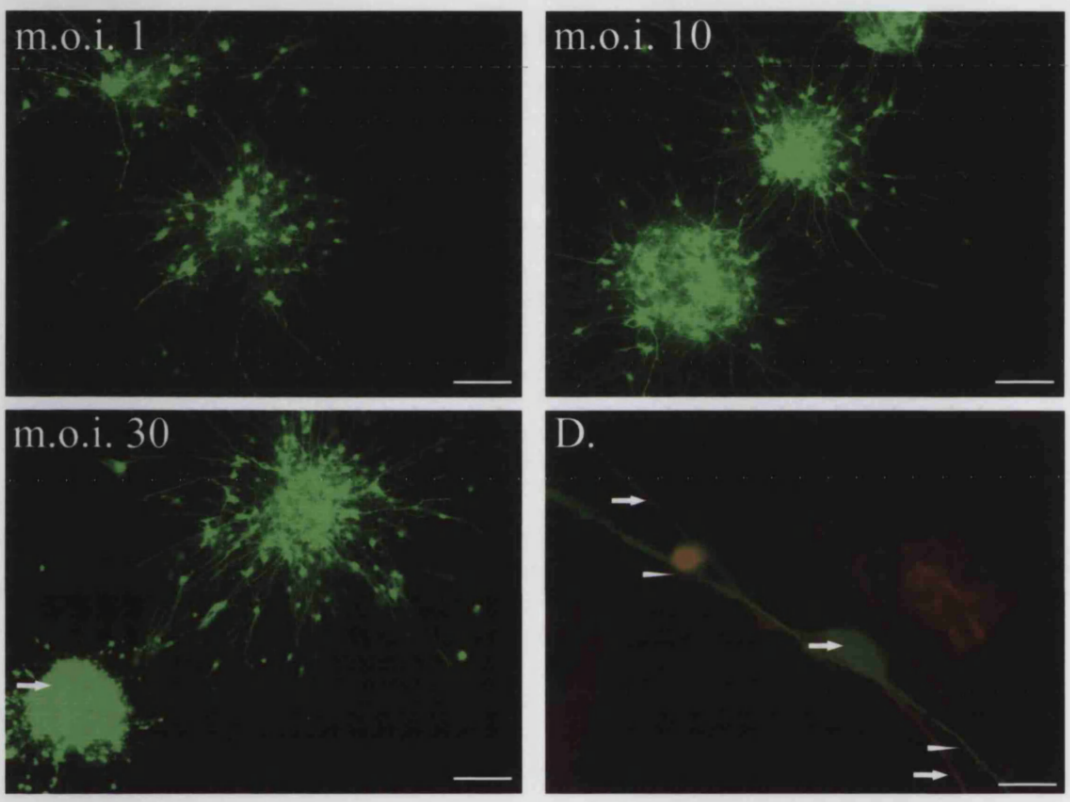


Figure 3-15: Transduced ^{mes}NS retain capacity to migrate on substrate. Following transduction at an m.o.i. of 1, 10 or 30, mesencephalic neurospheres were plated on PLL/Laminin coated glass cover slips and maintained for one day in differentiation media. Cells were fixed in 4%PFA and images were taken under an epifluorescent microscope with FITC filter. At all multiplicities tested transduced precursors formed long radial processes. Only at high viral amounts (m.o.i. 30) cytopathic effects became visible (arrow) and process formation was inhibited. Transduced cells migrated similar as untransduced precursor cells away from the neurosphere and differentiated into neural cell types in the absence of mitogens. (D) Immunocytochemical staining showing a high power magnification of a transduced ^{mes}NS that differentiated into a TuJ1 positive neuron (Alexa 546, arrow) likely to be migrating along the process of an infected radial glial process. Scale bar represents 100µm, 10µm in (D).

As shown for adeno-associated viruses, viral transduction may effect proliferation of the NSC, slowing or ceasing the continuous growth of transduced precursors even in the presence of growth factors (Wu et al., 2002). Due to the slow growth rate of ^{mes}NS, the proliferation effects were studied in cortical and striatal derived neurospheres that grow considerably faster and allow possible growth inhibiting effects to be observed more easily. However, as ^{ctx}NS were shown to be less susceptible to viral transduction with the highly disabled 1764/27-/4- vector, the less disabled construct RL1+/27+/4-pR19hGFPwpre was used for this study. Following transduction of ^{ctx}NS and ^{str}NS with RL1+/27+/4-pR19hGFPwpre at high multiplicity (m.o.i. 10) neurospheres were maintained in either proliferation or differentiation media. Reporter gene expression was detected three days post-transduction under an epifluorescent microscope (Figure 3-16). The reporter gene was stably expressed under proliferation and differentiation conditions. Under differentiation condition GFP expression appeared evenly distributed throughout the neurosphere and cells inside the sphere appeared to be infected at the same intensity as cells in outer cell layers. Maintaining infected neurospheres in the presence of FGF2 resulted in a graded gene expression within the spheres (Figure 3-16). While the centre of the sphere contained a high density of NSC strongly expressing the reporter gene, the outer cell layer contained less or no GFP expressing precursor cells. The intensity of GFP expression in the outer cell layer was also observed to be less strong compared to the core of the spheres. An explanation might be that the core of high GFP expression represented cells that were initially transduced. As infections were performed at high multiplicity the majority of NSC's were infected most likely with more than one virion. As neurospheres continue to grow in the presence of the mitogen from inside to outside, the daughter cells formed the outer layers of the sphere. Since HSV-1 remains episomal and does not integrate into the host genome only one daughter cell will inherit the genome in case that the mother cell was infected by a single viral particle, or the viral genomes are distributed on both daughter cells in case of multiple initial transduction events. With continuous proliferation of the NSC the viral genetic information will be further diluted and consequently, fewer cells in the outer cell layers will contain the recombinant episome. The result is the observed graded GFP expression under proliferating conditions. Indeed virally infected neurospheres further

increased in diameter when FGF2 was present and no significant differences in the growth rates were observed compared to uninfected neurospheres.

Reporter gene expression was stable under differentiation conditions for at least two weeks, the longest time point tested. Over this time frame no decrease in the number of reporter gene expressing cells or in the intensity of GFP expression was observed. A continuous passaging of infected neurospheres has not been tested due to the episomal character of HSV-1.

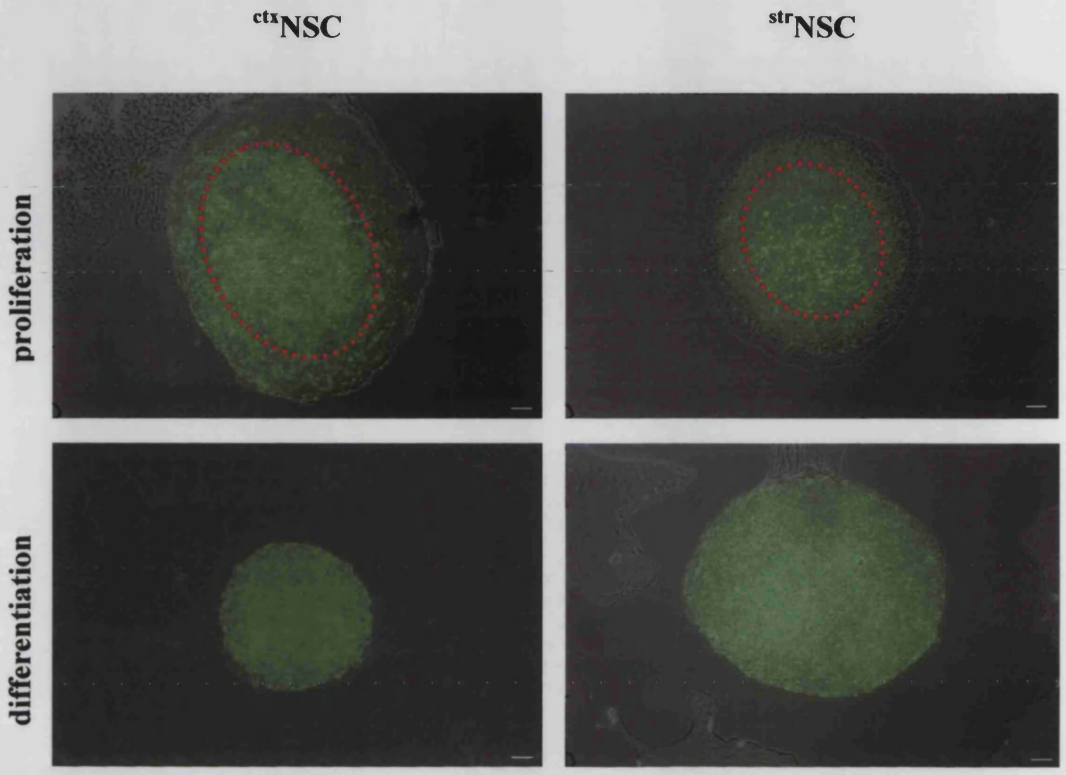


Figure 3-16: Viral transduction does not inhibit proliferation of neurospheres in growth factor containing media. Overlay of fluorescent and phase contrast images of cortical and striatal neurospheres infected at an m.o.i. of 10 with RL1+/27+/4-/pR19hGFPwpre. After transduction neurospheres were maintained for another two days in either proliferation media (containing 20ng/ml FGF2) or differentiation media (no FGF2). In the presence of FGF2 transduced neurosphere continued proliferating, but as HSV-1 remained episomal, not every daughter cell contained a viral genome. This resulted in a cell layer with reduced number of reporter gene expressing NSCs surrounding a core of originally transduced cells (dotted line) und proliferating conditions. This layer of reduced GFP expression was not evident under differentiation conditions. Scale bar represents 20µm.

3.3.2.5 Transduction with HSV-1 does not impair differentiation into the different neural cell types

A defining characteristic of neural stem cells is their ability to give rise to neurons and glial cells (Gage, 2000). NSC's grown as neurospheres remain multipotent even after continuous passaging. This makes them favourable as source for neural tissue (Svendsen et al., 1999). As viral transduction may affect this natural ability to differentiate into neuronal or glial cell types, whether infected NSC's retained this capacity was studied, considering two aspects: can infected neurospheres give rise to neural cell types, and into which neural cell type do infected NSC's differentiate upon serum withdrawal?

In order to address these questions cortical, striatal and mesencephalic derived neurospheres were infected with either the highly disabled construct 1764/27-/4-/pR19hGFP or the less disabled vector RL1+/27+/4-pR19hGFPwpre. Following transduction the neurospheres were plated on poly-L-lysine/laminin coated cover slips to take advantage of the cell migration assay. The transduced NSC's were differentiated for five days prior to fixation in paraformaldehyde. Neurons were identified by immunocytochemical labelling with the post-mitotic marker TuJ1 and glia by staining for GFAP reactivity (Figure 3-17 and Figure 3-18). Infected NSC's of all three neurogenic regions retained their ability to differentiate into neurons and glia. Virally infected NSCs preferentially differentiated into GFAP positive astrocytes, a phenomenon that appeared independent from the region of the NS or the disablement of the viral vector. Differentiation of transduced NSC into neurons was not completely abolished as several migrated TuJ1 positive cells expressing GFP were detected after infection with either construct in the three regions tested. However, the total number of transduced NSC's differentiating into neurons was different for the two viral constructs. NSC's infected with the highly disabled vector gave rise to more neurons than with the less disabled construct. This is particularly evident in ^{str}NS that naturally give rise to more neurons than cortical or mesencephalic neurospheres (Ostenfeld et al., 2002). This observation may reflect differences in vector cytotoxicity affecting neurogenesis of infected neural progenitor cells. The viral gene

product ICP27 or ICP 34.5 maybe toxic in neuronal progenitor cells resulting in an early cell death of transduced progenitors determined to become neurons while it obviously is non-toxic in glial progenitor cells that retained the capacity to differentiate into mature GFAP positive astrocytes. This toxic effect was not evident in the 27- mutant giving rise to plenty TuJ1/GFP double labelled neurons in striatal neurospheres. The low number of double labelled neurons in ^{ctx}NS was mainly due to the inefficient gene delivery to cortical precursor cells with 1764/27-/4-pR19hGFP as described earlier in this chapter. That transduced ^{str}NS mainly differentiate into astrocytes may reflect the predominance of glial progenitor cells in neurospheres expanded under the chosen culture conditions. Cytotoxic effects of viral transduction with 1764/27-/4-pR19hGFP on neuronal morphology became obvious at high multiplicities (m.o.i. 10). The somas of infected neurons were enlarged and neural processes often shortened or disintegrated. At the same multiplicity no cytopathic effects were observed in transduced NSC that had differentiated into GFAP positive glial cells (Figure 3-17).

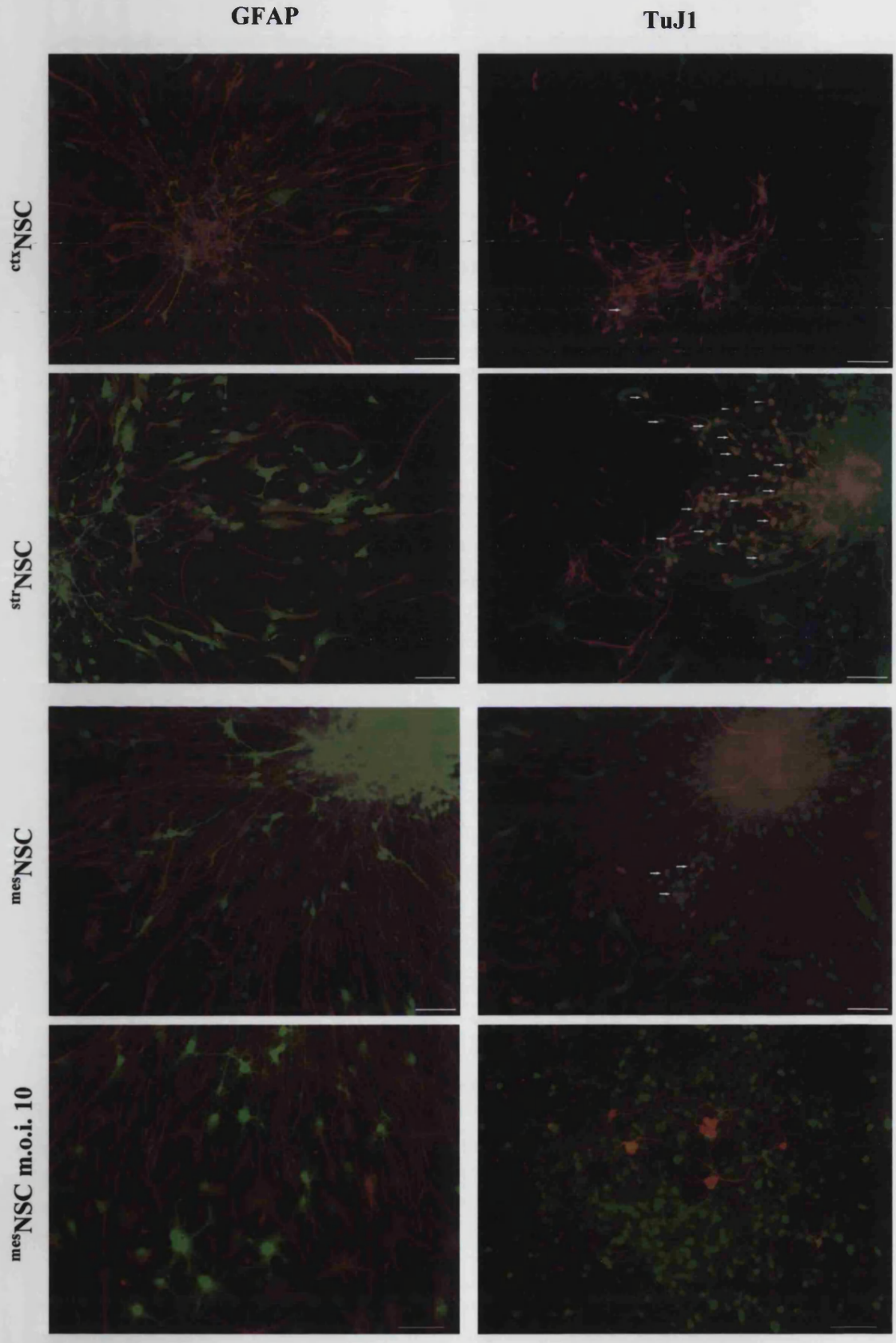


Figure 3-17: Neurospheres from different regions retain the ability to differentiate into astrocytes and neurons after transduction with 1764/27-/4-pR19hGFP. Cells were fixed and stained for TuJ or GFAP (Alexa 546, red), respectively. Independent of the neurogenic region, the transduced precursors predominantly differentiated into astrocytes. However, neurogenesis is not inhibited as shown by GFP/TuJ double labelled neurons (arrows). High multiplicities cause cytopathic effects in neurons visible in swollen cell bodies and disintegrated processes. Scale bars represent 50µm.

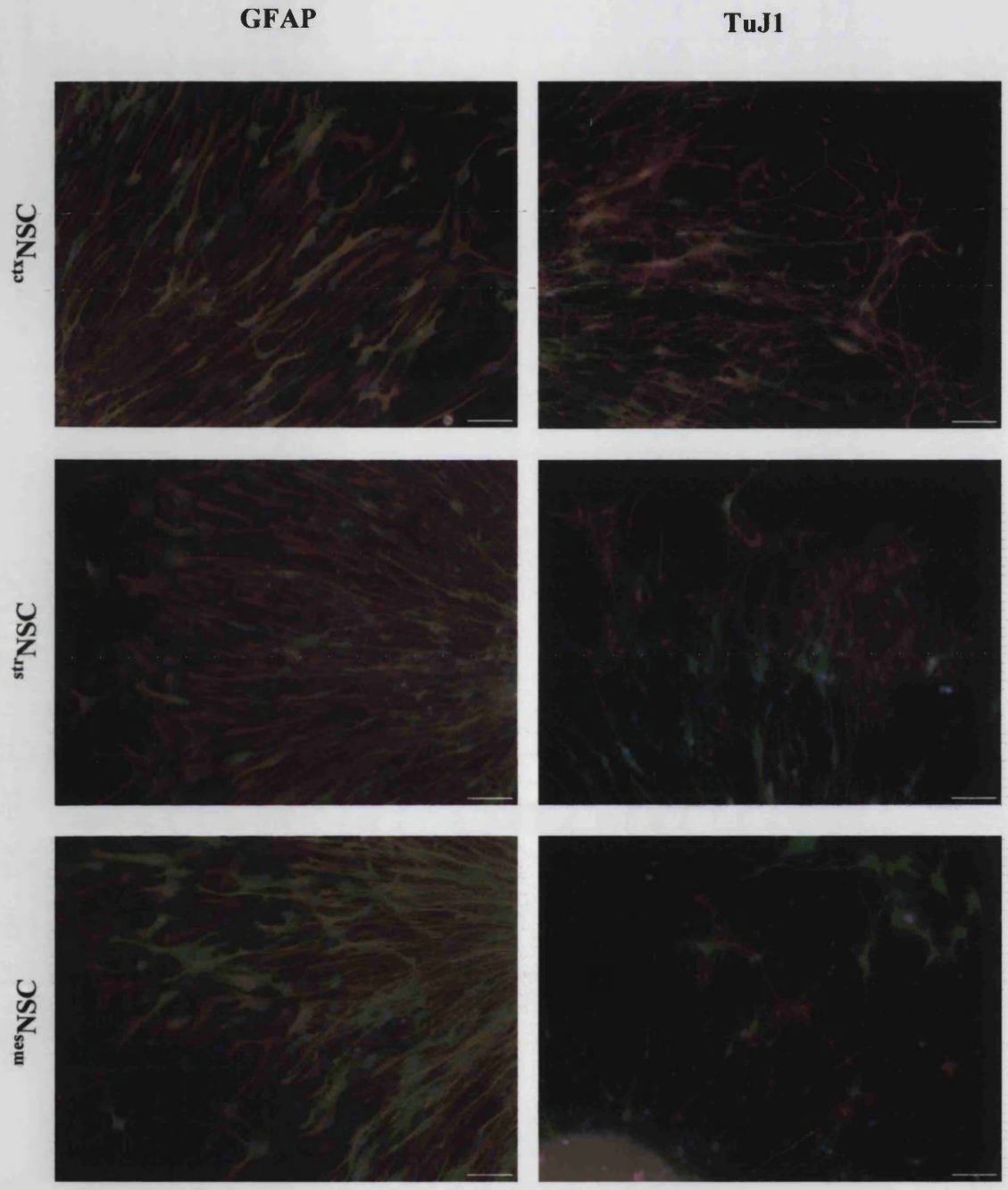


Figure 3-18: Neurospheres from different regions retain the ability to differentiate into astrocytes and neurons after transduction with RL1+/27+/4-pR19hGFPwpre. NSC's derived from the different neurogenic regions mainly differentiate into GFAP positive astrocytes. Although neurogenesis is not abolished as shown by the occurrence of TuJ1 positive neurons, viral transduction seems to have negative effects on neurogenesis of transduced precursor cells as only a low number of GFP/TuJ double labelled cells were detected. Scale bars represent 50µm.

3.3.2.6 Viral transduction does not induce premature differentiation

The previous section has shown that neural stem cells infected with the less disabled vector RL1+/27+/4-pR19hGFPwpre predominantly differentiate into GFAP positive astrocytes. It was suggested that the viral gene product ICP27 may inhibit differentiation of neuronal progenitor cells into post-mitotic neurons, most likely due to cytotoxic effects. On the other hand no inhibiting effects on gliogenesis were observed dependent of the neurogenic region the neurospheres were generated from. However, this does not exclude the possibility that viral transduction with the less disabled construct or that the expression of viral genes induce a premature differentiation of a common precursor cell into a glial phenotype. That viral transduction of neurospheres can induce an unexpected differentiation into GFAP positive astrocytes accompanied by a decrease in nestin expression even under proliferation conditions has been reported for adenoviral vector systems (Hughes et al., 2002; Falk et al., 2002) and was also observed with adeno-associated vectors (Wu et al., 2002). To address this possibility induction of GFAP expression was considered after viral transduction of neurospheres under proliferating conditions.

In the presence of the mitogen FGF2 neurospheres remain in an undifferentiated state and the majority of neural stem cells were expected to be nestin positive. Striatal neurospheres infected with either the highly disabled vector 1764/27-/4-pR19hGFP or the less disabled vector RL1+/27+/4-pR19hGFPwpre showed indeed strong immunoreactivity for nestin when maintained in proliferation media and fixed one day after viral transduction (Figure 3-19E and F). More than 90% of the radial fibers were nestin positive at this time point. Although the majority of GFP expressing cells (>80%) were co-localising with nestin, some transduced progenitor cells that migrated onto the substrate were not immunoreactive for the neurofilament marker. GFP expression was localised in the cell bodies and radial processes of the nestin positive cells.

In order to study if transduction with RL1+/27+/4-pR19hGFPwpre induces differentiation of neural progenitor cells into astrocytes even under the continuous

presence of growth factors, the neurospheres were plated on PLL/Laminin coated cover slips and a cell migration assay performed as described before. Individual transduced precursors and cells derived from NSC's migrated were characterized three days post-transduction for their antigenic identity. Control cultures were treated with serum-free media instead of diluted virus stock during infection. Following transduction the neurospheres were maintained in media containing FGF2 (20ng/ml). Surprisingly, a significant number of GFAP positive astrocytes (Figure 3-19 A and B) were found in both virus treated and control cultures, suggesting that some of the cells derived from migrating NSC's have differentiated despite the continuous presence of FGF2. However, the majority of neural precursor cells in control and virally infected cultures stained positive for nestin. By immunocytochemical staining an increased number of GFP expressing cells co-localised with GFAP was not observed. Most of the transduced NSC's remained undifferentiated as shown by co-localisation with nestin (Figure 3-19C). To quantify differences in GFAP expression from neurospheres transduced with RL1+/27+/4-pR19hGFPwpre or SFM and maintained under proliferation conditions, western blot analysis was performed (Figure 3-19 G). Although both neurosphere cultures express the astrocytic marker even under proliferation conditions, no differences in the expression levels were detected between virally and non-virally infected samples. Therefore, it was concluded that transduction with disabled HSV-1 did not induce a premature maturation of uncommitted NSC's. The observation that some of the NSC's differentiated under proliferation conditions independently of a viral transduction event, maybe the result of insufficient supply with growth factor over three days cultivation period.

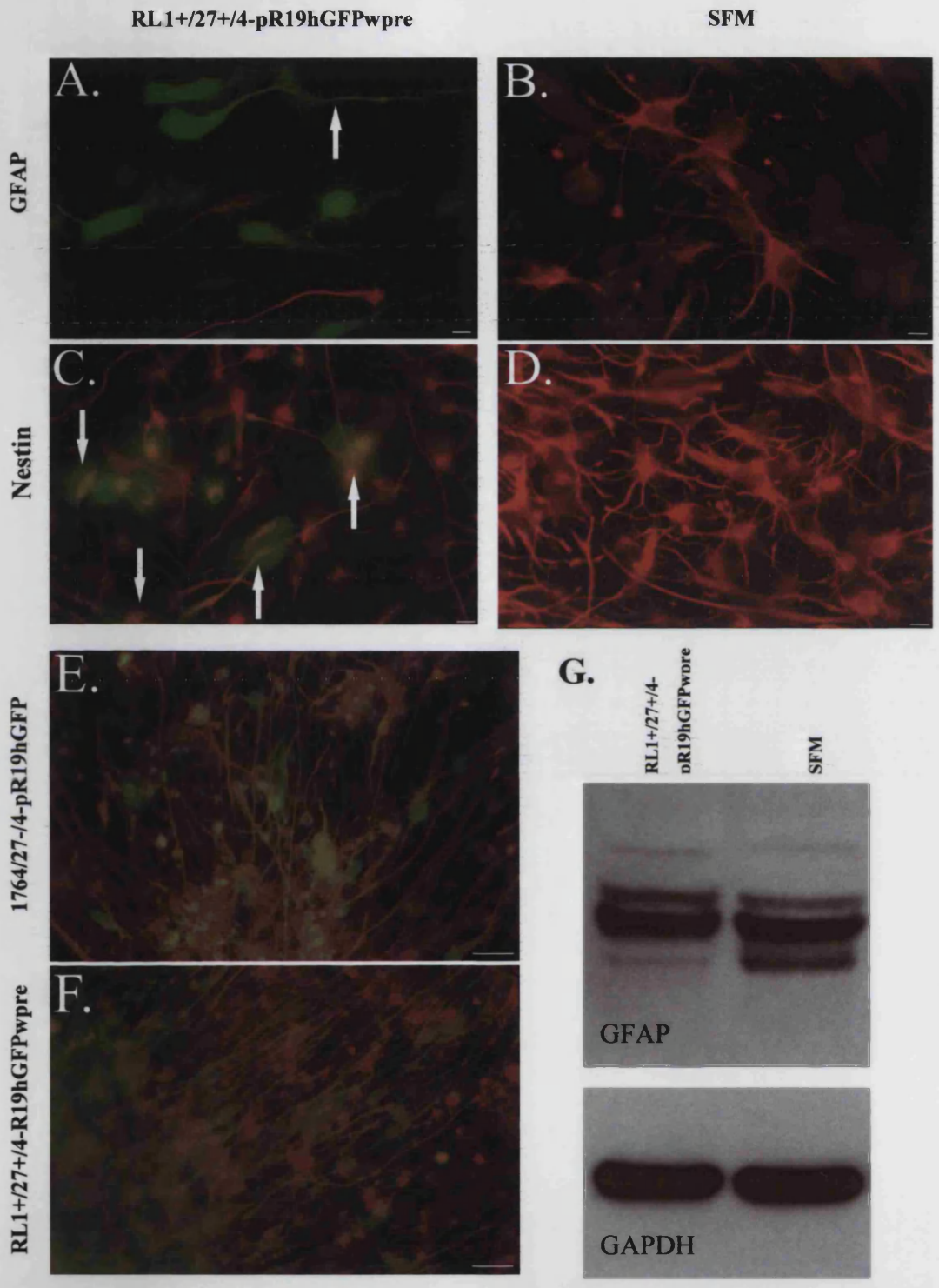


Figure 3-19: Viral infection does not induce premature differentiation of NSC's. ^{str}NSC were infected with RL1+/27+/4-pR19hGFPwpre (A to D, F) or 1764/27-/4-pR19hGFP (E) and maintained in the presence of FGF2 for another one (E and F) or three days (A to D) prior to fixation with paraformaldehyde. (A) and (B) GFAP expressing cells (Alexa 546, red) were detected in control and virally infected cultures. Although some transduced NSC's have differentiated into GFAP positive astrocytes (arrow), the majority of GFP expressing cells did not co-localise with the astrocytic marker. (C) and (D) Nestin (Alexa 546, red) was expressed in the majority of control and virally infected cultures (arrows). (E) and (F) One day after infection most of the GFP expression co-localised with nestin expression in the radial fibers emerged from the neurospheres. (G) Western blot analysis confirming that GFAP expression was not upregulated in ^{str}NS that had been infected with RL1+/27+/4-pR19hGFPwpre and maintained in the presence of FGF2 for three days after transduction.

3.3.2.7 Gene delivery to human neural stem cells

Although this study preliminarily focused on the use of rodent neurospheres, neurospheres derived from human tissue were also tested for transduction with replication-deficient HSV-1. Mesencephalic neurospheres derived from human tissue (^{mes}hNS) were previously prepared and had been passaged using a chopping method that allowed extended growth of otherwise senescent human neurospheres (Svendsen et al., 1998).

^{mes}hNS were infected as whole neurospheres with the highly disabled construct 1764/27-/4-pR19hGFP at an m.o.i. of 10. Using the migration assay, transduced ^{mes}hNS were subsequently plated on poly-L-lysine/laminin coated substrate and maintained in differentiation media. Reporter gene expression was visualised under an epifluorescence microscope (Figure 3-20) showing efficient gene delivery to ^{mes}hNS. As described before for rodent NSC's, the infected human NSC's also retain the ability to emerge radial processes and neural precursor cells migrate onto the substrate to differentiate in distance of the neurosphere. As shown by immunocytochemical staining for the neuronal marker TuJ1, infected human NSC's can still differentiate into post-mitotic neurons with long neural processes without any evident cytopathic effects (Figure 3-20). Transgene expression in ^{mes}hNS is stable and could be detected for up to 14 days, the longest time point tested.

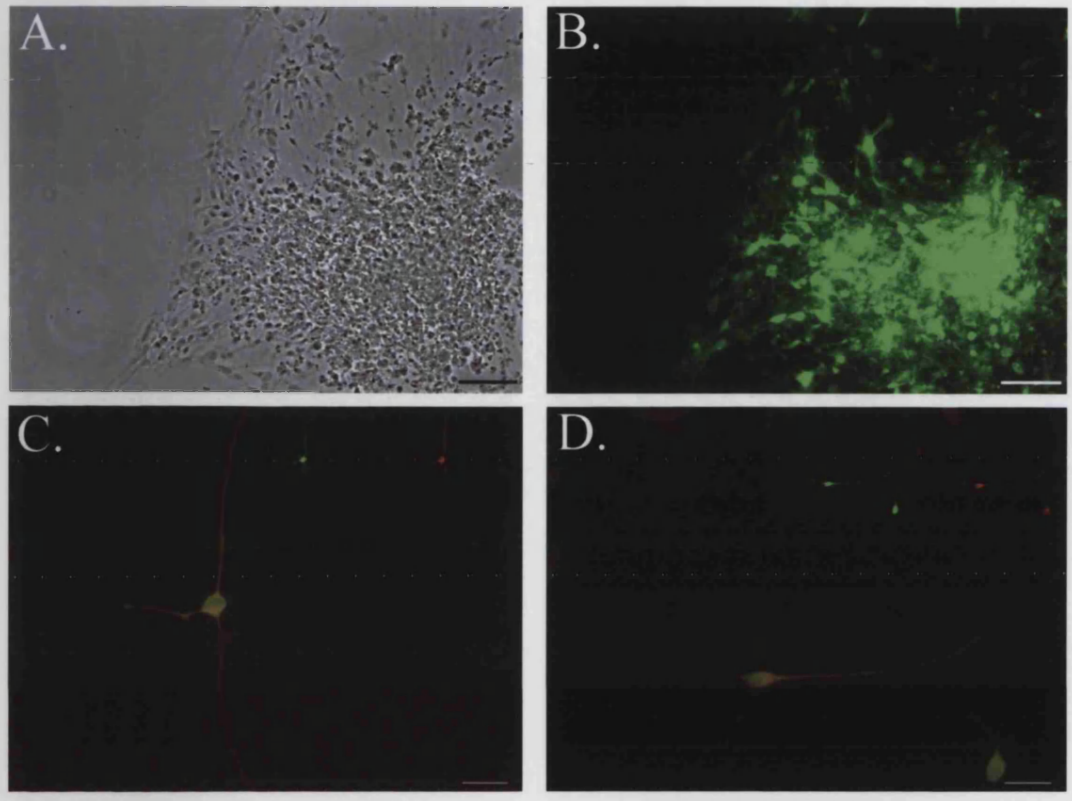


Figure 3-20: Transduction of human neurospheres generated from embryonic mesencephalic tissue. Mesencephalic derived neurospheres were previously isolated and prepared from human foetal tissue and passaged using a unique tissue chopping method. Neurospheres were infected with 1764/27-/4-pR19hGFP at an m.o.i. of 10 and subsequently plated on PLL/Laminin coated cover slips. Infected ^{mes}hNS formed radial processes and precursor cells migrated onto the substrate in distance of the original sphere as shown in (A) a phase contrast image. (B) GFP expression three days post-transduction demonstrating high gene delivery efficiency to hNSC's, (C), (D) Infected NSC's retain their ability to differentiate into TuJ1 positive neurons as confirmed by immunocytochemical staining. Merged images for TuJ1 (red) and GFP in the same neurons. Insets show unmerged images. Scale bars represent 100μm in (A) and (B), 10μm in (C) and (D).

3.3.2.8 Transplantation of virally infected neurospheres

Neurospheres have also emerged as promising cellular vehicle for *ex vivo* gene transfer approaches. The approach aims to expand and genetically manipulate neural stem cells *in vitro*, prior to transplantation to the CNS. Thus, safety concerns about direct administration of virus are diminished as the genetically modified cells could be tested for any remaining virus prior to transplantation. It has been well established that neural stem cells can survive, migrate and functionally integrate into the host circuitry after intracerebral transplantation (reviewed by Lindvall, 2003). Other groups have also demonstrated that neurospheres transduced *a priori* with adenoviral (Sabate et al., 1995; Corti et al., 1999) and lentiviral vectors (Ostenfeld et al., 2002) retain this ability. Here, the aim was to establish if rodent neurospheres transduced with replication-deficient HSV-1 survive and express the transgene after intracerebral transplantation.

The experimental rationale was to transplant ^{mes}NS that were transduced two days prior grafting with the less disabled vector RL1+/27+/4-pR19hGFPwpre into the striatum of adult rats and to determine reporter gene expression in the graft three days and three weeks after grafting (Figure 3-21A). ^{mes}NS were freshly prepared and expanded for ten days before infection with RL1+/27+/4-pR19hGFPwpre at an m.o.i. of 1. Strong reporter gene expression was confirmed two days after transduction (Figure 3-21B) and an aliquot of the neurospheres was plated on PLL/Laminin coated cover slips to stain for the presence of TH expressing neurons (Figure 3-21B). Unpassaged neurospheres were used in this study for optimal cell survival and a low number of TH expressing cells were identified, most likely residual primary dopaminergic neurons from tissue preparation. The transduced mesNS were transplanted as whole neurospheres (diameter about 200μm per sphere) at two injection sites unilateral into the striatum of rats. 10,000 viable cells were transplanted per injection site with three animals per group. As the same rat strain was used for preparation of neurospheres and as host for grafting, immunosuppression of the animals was not required. The animals were sacrificed three days or three weeks after transplantation, the brains removed and coronal sections prepared.

Three days after grafting evidence was found of GFP expressing cells around the injection site (Figure 3-21C). Due to the surgical procedure, significant cell damage was observed around the grafting site. GFP expression was only observed in the vicinity of the graft at this time point in the form of spherical deposits. Only a few reporter gene expressing cells had migrated short distances from the injection site into the host tissue. A significant upregulation of GFAP (Figure 3-21D) and nestin (Figure 3-21B) expression was found around the graft most likely due to microglia infiltration. Physical stress and damage caused by the surgical procedure may have caused this host response. Interestingly, evidence of cell bodies staining for TH within the graft and around the injection site (Figure 3-21F) was found. These TH positive cells had the ability to migrate into the striatum of the host (Figure 3-21G) and also formed processes into the host tissue (inset in Figure 3-21G). As dopaminergic cell bodies are localised in the substantia nigra and only the processes project into the striatum, the detected TH positive cells probably originated from the graft. No TH positive cell bodies were found in the striatum contralateral to the injection site. However, the TH expressing cell bodies did not co-label with the reporter gene GFP suggesting that they were not virally transduced. As shown before in this chapter, primary dopaminergic neurons were generally rather difficult to transduce with disabled HSV-1 and it is likely that the detected TH positive cells in the host striatum were actually untransformed, residual primary dopaminergic neurons. Therefore, it cannot be concluded from these data if viral transduction affected the migration capacity of the NSC as no GFP expressing cells were detected at any further distance to the grafting site at this time point.

Under the tested conditions no long term GFP expression was observed in the grafted neurospheres. Three weeks after transplantation no GFP expression could be detected in the striatum of grafted animals (Figure 3-21H). This might be the result of poor survival of virally transduced neurospheres and/or due to rejection as result of the inflammatory response that was observed in the three day time point. A similar lack of long-term survival was also found in other studies (Svendsen et al., 1996).

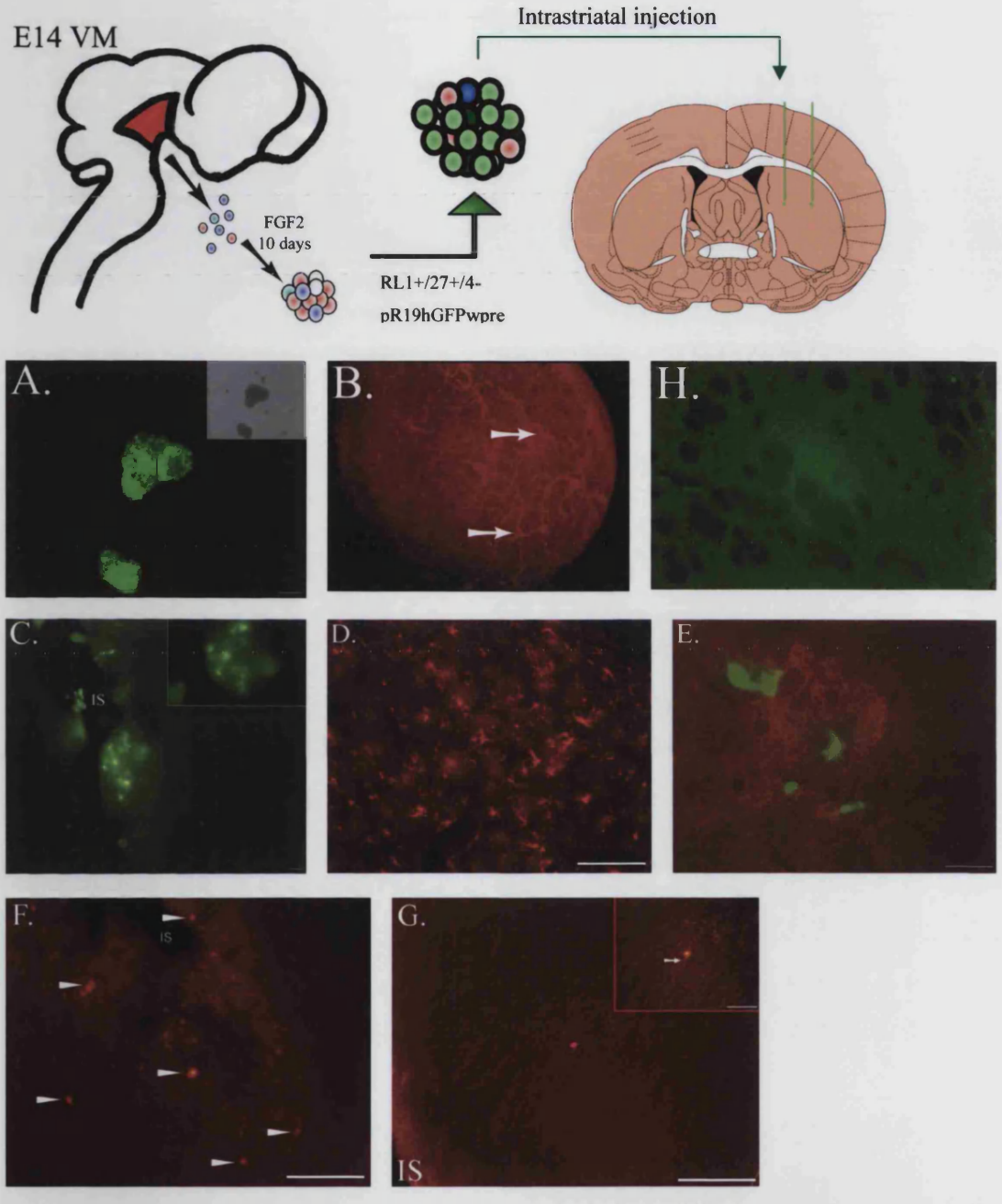


Figure 3-21: Transplantation of transduced neurospheres. ^{mes}NS were expanded for 10days prior viral transduction with RL1+/27+/4-pR19hGFPwpre. (A) Infected neurosphere showed strong GFP expression prior to transplantation. 10,000 cells were transplanted into two sites of the striatum of adult rats. Animals were terminated 3 days and 3 weeks after transplantation. (B) An aliquot of the transduced neurospheres was plated on PLL/laminin coated glass coverslips and stained for TH expression (arrow). (C) Clusters of GFP expressing cells were found around the injection site (IS). (D) GFAP and (E) nestin expression were upregulated around the injection site most likely due to surgical tissue damage. (F) TH staining revealed TH expressing cell bodies around the grafting site (arrowheads) and (G) some of the TH expressing cells were found in distant to the graft within the host striatum. Inset shows TH+ve process emerging into the tissue. (H) No GFP expression was detected 3 weeks after transplantation. Scale bars: 100µm in A, C, E, F; 50µm in B, D and inset of F.

3.3.3 HSV-1 as a gene delivery vector to endogenous neural stem cells in the subventricular zone of the CNS

In the adult mammalian forebrain new neurons are continuously generated from progenitor cells that are localised in the subventricular zone (SVZ) (Alvarez-Buylla and Garcia-Verdugo, 2002). Remarkably, neural progenitors can migrate long distances from the site of birth in the SVZ to the olfactory bulb, where they fully differentiate into mature neurons (Lois and Alvarez-Buylla, 1994). In order to study behaviour of endogenous neural stem cells or to deliver potentially therapeutic genes to the SVZ, viral vectors are particularly useful for *in vivo* gene delivery to neural stem cells in this region. Previously it has been demonstrated that cells of the SVZ can efficiently be transduced using adenoviruses (Yoon et al., 1996; Benraiss et al., 2001) and adeno-associated vectors (Davidson et al., 2000) after intraventricular injection. Here, replication defective HSV-1 was used to infect adult precursor cells in the subventricular zone and their migration followed to the olfactory bulb.

To test whether replication-deficient HSV-1 could label cells localised within the SVZ, the less disabled vector was injected into the lateral ventricles of adult rats (older than 6 months). $5x10^6$PFU of RL1+/27+/4-pR19hGFPwpre were injected bilaterally into three animals and the animals terminated four days after injection. The brains were removed, coronally sliced into 40μm rostral-caudal sections on a freezing microtome and reporter gene expression monitored under an epifluorescent microscope (Figure 3-22). GFP expressing cells were found in the walls of the lateral ventricles around the injection site (Figure 3-22C), as well as in distant sections rostral (Figure 3-22A) and caudal (Figure 3-22B) of the injection site. Thus, the highly concentrated virus applied into the lateral ventricles, diluted in the cerebrospinal fluid (CSF) and was transported with it throughout the ventricle system to infect cells of the ventricle wall at a distance from the actual injection site. Little parenchymal reporter gene expression was noted. No GFP expression was observed at this time point in the striatum or septum, suggesting that viral penetration outside the subependyma was minimal. A significant higher number of labelled cells were found in the ventricle wall facing the striatum rather than septum.

It has been shown before that adenoviral vectors injected directly into the SVZ of the lateral wall labelled precursor cells that retained the ability to migrate to the olfactory bulb where they expressed the reporter gene LacZ (Yoon et al., 1996). To study if a similar result can be obtained with HSV-1 vectors, highly disabled 1764/27-/4-pR19LacZ was injected directly into the SVZ rather than into the ventricle and LacZ expression observed in the olfactory bulb (Figure 3-22) three days after virus injection. In all animals (n=3) we found strong LacZ expression around the injection site around the dorsolateral horn of the ventricle and also detected LacZ expressing cells in the OB. In the OB LacZ expressing cells were mainly observed in chains that may reflect the migration of transduced progenitors within the olfactory stream. The reporter gene expressing cells showed a rather globular cell shape without visible processes, suggesting that a differentiation into mature neuronal cell types had not occurred at this time point. However, the lack of LacZ product localised in processes may be a problem related to the use of LacZ as reporter gene since it has been shown before that β-gal was mostly defined to the cell perikaryon of these cells (Yoon et al., 1996).

To further characterize the cellular identity of the transduced cells within the SVZ, immunohistochemical staining for GFAP and nestin was performed. The cellular composition and architecture of the adult mouse SVZ has been characterized before (Doetsch et al., 1997) and the SVZ-ependymal layer contains at least four different cell types (Alvarez-Buylla and Garcia-Verdugo, 2002). Most notable is the population of dividing astrocytes (B cells) that were identified as the *in vivo* primary precursors in this region and act as stem cells *in vitro* (Doetsch et al., 1999). B cells interact closely with ependymal cells which separate the SVZ from the lateral ventricle. Occasionally, an SVZ astrocyte extends a process between ependymal cells to contact the ventricle lumen. Whereas ependymal cells have long cilia, some B cells contain a single short cilium (Doetsch et al., 1999).

As shown in (Figure 3-22A) GFP expressing cells were found in the subependymal layer of the SVZ in a zone comprising about two to three cell layers of the lateral wall. Unfortunately, the ependymal layer came off during tissue processing and thus,

no conclusion can be made regarding transduction efficiency of ependymal cells. However, sections containing the ependymal layer as ribbon floating within the ventricle showed strong reporter gene expression suggesting that both, ependymal and subependymal layers, were transduced. Immunohistochemical staining for GFAP revealed as expected a high number of astrocytes within the subependymal layer (Figure 3-23B and C). Some of the astrocytes were expressing the reporter gene and had globular (arrows in Figure 3-23B) or branched (arrow head in Figure 3-23B) cell morphologies. Interestingly, some of the virally transduced astrocytes had short single processes that emerged into the lumen of the ventricle (Figure 3-23D). As mentioned above, these may be the astrocytes giving rise to faster dividing neural progenitors that had been identified by others as the neural stem cells of the SVZ (Doetsch et al., 1999). Immunohistochemical staining for the neurofilament marker nestin showed a ribbon of nestin positive cells along the wall of the lateral ventricle (Figure 3-23D and E). Within this layer of nestin positive cells GFP expressing cells were localised. As shown in Figure 3-23E some the transduced cells appear to co-label with the neurofilament marker.

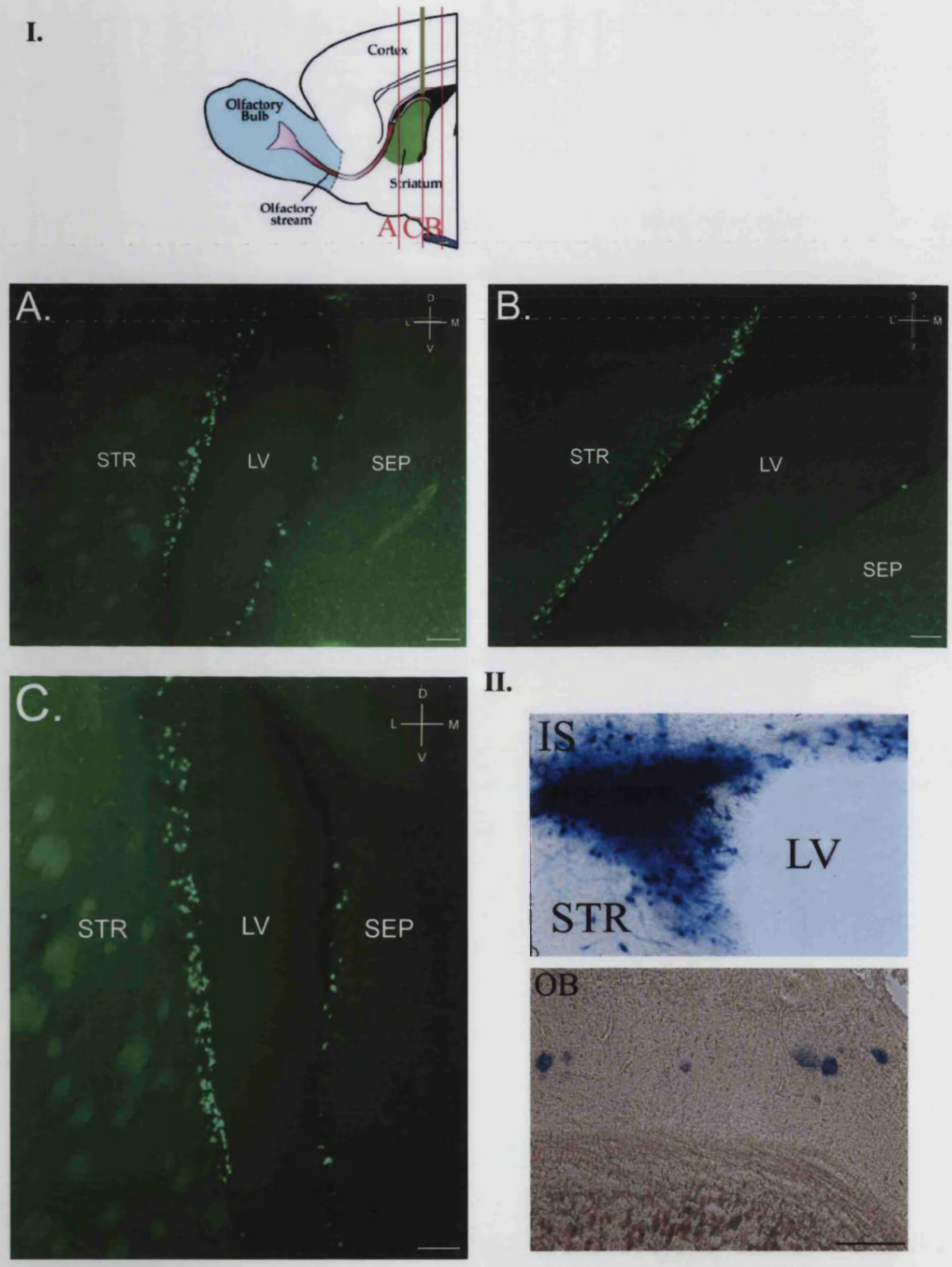


Figure 3-22: Disabled HSV-1 transduced cells of the subventricular zone (SVZ). As shown in the illustration progenitor cells born in the SVZ migrate via the migratory stream to the olfactory bulb where they differentiate into neurons. I: Replication deficient HSV-1 RL1+/27+/4-/pR19EGFPwpre was injected into the lateral ventricles and gene expression monitored in (A) rostral, (B) caudal and (C) sections to the injection site. GFP expressing cells were detected in the wall of the SVZ throughout the entire lateral ventricle (LV), here shown in representative sections. No GFP expression was found in the septum (SEP) or striatum (STR). II: Direct injections of 1764/27-/4-/pR19LacZ into the SVZ (IS). LacZ expression was detected in the olfactory bulb (OB) three days after virus injection. D=dorsal, V=ventral, M=medial, L=lateral. Scale bar represents 100µm.

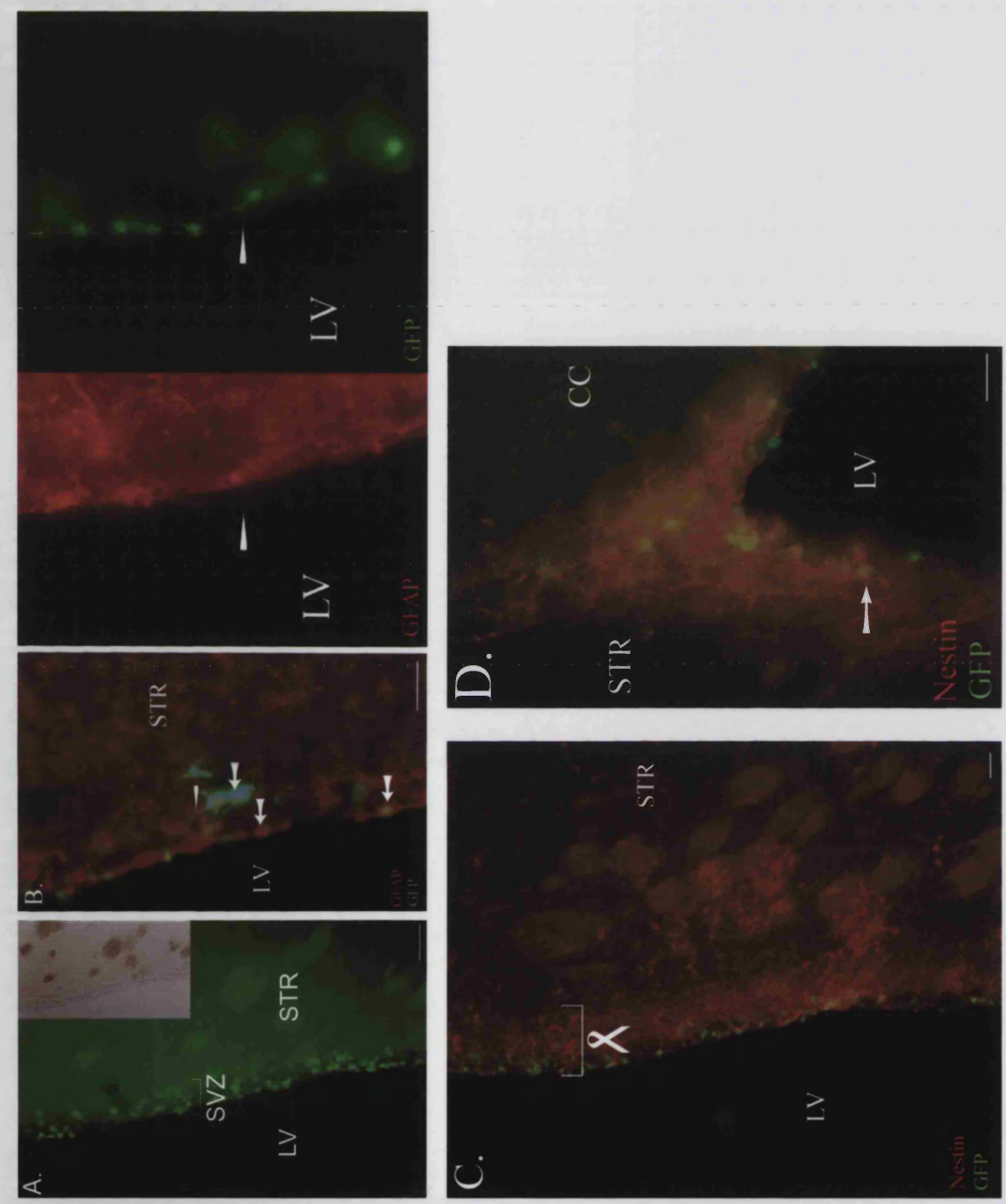


Figure 3-23: Characterization of transduced cells in the SVZ. (A) GFP expression was detected in cells of the subependymal layer of the ventricle wall. Inset in (A) is a phase image to confirm no cytotoxic effects of viral infection in the wall of the LV. (B) Merged image of GFP expression and GFAP expression. Some of the transduced cells in the SVZ co-labelled with GFAP (arrows). GFP/GFAP double labeled cells differed in cell size and morphology. Some of the double labelled cells were small and globular, others had a large cell body with processes (arrowhead). (C) Along the lateral nestin positive cells were found as a ribbon (about 150µm) along the wall of the lateral ventricle. (D) Some of the nestin positive cells within this ribbon were expressing GFP (arrow). LV=lateral ventricle, STR=striatum, SVZ=subventricular zone, CC=corpus callosum. Scale bars represent 100µm in A and 50µm in B-D.

3.4 Discussion

In this chapter it was demonstrated for the first time that replication-deficient HSV-1 can efficiently deliver foreign genes to neural precursor cells of the CNS. Two different disabled viral vector systems with also different promoter cassettes were tested for their suitability as gene delivery systems to neural progenitor and neural stem cells *in vitro* and *in vivo*.

Neural progenitor cells follow phenotypic changes in vitro

Since the overall aim of this study focuses on dopamine neuron development, embryonic tissue was chosen at a developmental stage that corresponds to the beginning of dopamine neuron development. In the rat embryo, midbrain dopamine development occurs between embryonic day 11 (E11) to E15 (Hanaway et al., 1971; Lauder and Bloom, 1974). Characterized by the expression of tyrosine hydroxylase it has been shown that mesencephalic dopamine neurons begin to differentiate at E12.5 (Specht et al., 1981). Dissections of embryos before the gestational stage E14 are technically difficult to perform and only a low number of mesencephalic progenitors can be obtained.

In the first section of this chapter the isolated neural progenitor cultures were characterized regarding their phenotypic identity. Of particular interest for this study were mesencephalic derived progenitor cells that give rise to dopaminergic neurons. Depending on the age of the culture about 62% of the cells were neuronal and 10% of the cells positive for the dopaminergic marker TH. The proportion of dopamine neurons is higher than in other studies reporting about 5% of TH positive cells from rat mesencephalic tissue cultured under similar conditions (Cheung et al., 1997). However, this might be the result from variations in the gestational age, dissection methods, culture conditions, and possibly the use of different immunogenic reagents. Particularly primary mesencephalic progenitor cultures showed a high variability among the preparations regarding cell viability.

Although, growth factor free, differentiative culture conditions were used in this study, it was shown that the progenitor cells undergo a time dependent maturation *in*

vitro. Particularly cortical and mesencephalic neural progenitors contained a high proportion of undifferentiated, nestin positive progenitors at early culture time points that later mainly gave rise to neurons since the culture conditions did not support glial differentiation. Whether these undifferentiated, nestin positive cells were neural stem cells or fate committed neuroblasts cannot be explained from these data. However, cells were observed with weak immunoreactivity for nestin that also had morphological characteristics of immature neurons suggesting that a commitment towards the neuronal lineage has already occurred.

Hence, these neurogenic progenitor cultures present a potential *in vitro* system to study the effects of exogenous factors on dopamine neuron development.

Less disabled HSV-1 vectors efficiently express in neural progenitor cells but on the expense of neuronal cell loss

In order to establish the conditions determining transduction efficiency, effects of the maturation state of the neural progenitor culture on viral transduction were considered. Infection of more mature mesencephalic progenitor cultures resulted for both vector systems tested in significant higher gene delivery efficiency than transduction at earlier time points. Indeed, other studies have also shown that transduction efficiency with the highly disabled construct depends on the age of primary cultures (Lilley, 2000). However, for the overall aim of this study it was not practical to use fully differentiated neural cultures. In order to induce a dopaminergic phenotype from neural precursor cells, the developmental stage of the primary tissue used for preparation of progenitor cell cultures is important. As progenitor cells mature in culture, recombinant genes need to be delivered at early culture time points to progenitor cells which are still plastic and able to adapt different phenotypes. Therefore, transduction efficiency was studied in progenitor cells infected on 1 DIV although significantly higher gene delivery rates were found at later time points. At this early time point reasonable gene delivery was only achieved using the less disabled construct RL1+/27+/4-pR19hGFPwpre, while the highly disabled backbone 1764/27-/4-pR19hGFP gave an inadequate transduction efficiency.

In vitro gene transfer studies to neuronal cultures of the CNS using HSV-1 have previously been limited by vector cytotoxicity. Reduction of immediate early gene expression in replication-incompetent HSV mutants resulted in decreased cytotoxicity (Johnson et al., 1992; Johnson et al., 1994). Infectious viral particles may cause cytopathic effects in transduced neurons due to residual viral gene expression. However, non-infectious viral particles, as well as cell debris from the producer cell line present in the virus stock, may also contribute to cell death in primary neuronal cell cultures (Ho et al., 1995). To reduce the amount of components from the producer cell line and thus, to increase the quality of the viral stock, all viruses used in this study were clarified in two subsequent filtration steps. Virus cytotoxicity was assessed for the less disabled construct RL1+/27+/4-pR19hGFPwpre in neural progenitor cells. Virally induced cytopathic effects were concentration dependent and more evident upon infection of early cultures rather than more mature primary neuronal cells. It appeared contradictory that a significant loss in the total number of neurons was observed in mesencephalic progenitor cultures, but that the number of TH expressing dopaminergic neurons was unaffected by viral transduction. Neuronal cell loss may occur either after infection of post-mitotic differentiated neurons or alternatively, viral transduction of undifferentiated neuroblasts abolishes further differentiation into a mature phenotype. Considering minimal cytotoxic effects in more mature cultures and no effects on primary dopamine neurons, it appears that immature neuroblasts are more vulnerable to the viral insult. As shown in this study and by others (Bouvier and Mytilineou, 1995), not many undifferentiated dopamine neuroblasts were present at the chosen gestational stage, and the serum and growth factor free culture conditions did not support their proliferation. Hence, virally transduced neuroblasts are mostly of non-dopaminergic fate and due to the low susceptibility of mature dopaminergic neurons to infection with disabled HSV-1, the number of TH expressing cells remained unaltered even at high multiplicities. Nevertheless, the low transduction efficiency of mature dopaminergic neurons *in vitro* is surprising since high susceptibility of these neurons for this replication-deficient HSV-1 vector was demonstrated *in vivo* (Lilley et al., 2001).

Vector cytotoxicity and reporter gene expression may explain the neuronal cell loss observed in this study. HSV-1 amplicon vectors expressing enhanced green

fluorescent protein (EGFP) induce apoptosis in primary cortical neuronal progenitor cells (Detrait et al., 2002). In preliminary work comparing three different reporter genes, enhanced GFP, hr GFP and LacZ, we have found similar results regarding reporter gene toxicity in primary neuronal cultures. Primary cortical and mesencephalic neural progenitor cells infected at the same multiplicity with the RL1+/27+/4- backbone expressing either of the reporter genes in the same promoter cassette, showed reporter gene dependent cytotoxic effects: EGFP>>hGFP≥LacZ. At the same gene delivery rate, EGFP expressing neuronal cultures contained a significant higher number of rounded neurons without processes two days post-transduction compared to hGFP or LacZ expressing cultures. Significantly more EGFP expressing cells detached from the cell culture surface and were lost prior to cell fixation resulting in a reduced number of transgene expressing cells. Cytotoxic effects between hGFP and LacZ expressing neuronal cultures were less evident. Since the antigenic identity of the transduced cell was to be determined in immunoyctochemical staining, it was decided to use hGFP expressing viral vectors for *in vitro* studies. However, cytotoxic effects of accumulated hGFP protein may contribute to neuronal cell loss in this study.

Highly and less disabled vectors efficiently deliver transgenes to neurospheres

In the next section of this chapter gene delivery to neural precursor cells expanded as neurospheres was considered. It was demonstrated that replication-deficient HSV-1 vectors efficiently deliver to rodent and human neurospheres. Since work shown in following chapters is based on the delivery of recombinant genes of rodent origin, it was decided to perform all subsequent work with neurospheres derived from rats.

Significant differences in gene delivery efficiency were demonstrated for the highly disabled construct 1764/27-/4-pR19hGFP in neurospheres. Even at low multiplicities the vector delivered with high efficiency and most notably, without causing any obvious cytotoxic effects or interfering with the stem cell nature of the infected cells. Transduced precursor cells retain their ability to self-renew and to differentiate into glial or neuronal cell types. Although gene delivery was more efficient with the less disabled construct RL1+/27+/4-pR19hGFPwpre at low multiplicities, reduced

neurogenesis after infection at higher multiplicities was found. Cytotoxic effects make this vector for this reason more favourable for infections with minimal virus concentrations.

Next it was asked if neuronal loss may partly be explained due to glial differentiation of transduced precursor cells. A premature differentiation of HSV-1 infected precursor cells into GFAP positive astrocytes was not observed as described by other groups using adenoviral vectors (Hughes et al., 2002; Falk et al., 2002). However, this phenomenon described for adenoviral vectors may only occur in a subpopulation of already fate committed progenitor cells and was not observed when transgene expression was targeted to a selective population of uncommitted neural stem cell (Keyoung et al., 2001).

Viral transduction could be blocked in the presence of heparin, most likely by competing for heparan sulphates as receptor for viral attachment. Other studies had identified heparan sulphate as receptor for HSV-1 attachment in endothelial cells (Herold et al., 1994), and primary neural progenitor cells (Immergluck et al., 1998) and virus adsorption could be inhibited in the presence of heparin or by enzymatic digestion of cell surface heparin sulphates (WuDunn and Spear, 1989; Immergluck et al., 1998). Heparan sulphate proteoglycans contain a plethora of members that are expressed in the developing and adult CNS (reviewed by Yamaguchi, 2001) and were also found on neural precursor cells expanded *in vitro* (Brickman et al., 1995; Hagihara et al., 2000). Hence, viral attachment is also in neurospheres most likely facilitated by heparan sulphates.

Interestingly, transduction with the vectors indicated region-specific differences between expanded precursor cells derived from distinct neurogenic regions. With the three viral backbones tested a similar region-specific increase in gene delivery efficiency was found: ^{ctx}NS<^{mes}NS<^{str}NS. These region specific differences were found in rodent neurospheres and also observed in neurospheres derived from different neurogenic regions of human tissue. It is noteworthy that a similar expression pattern was also observed with the highly disabled 1764/27-/4-pR19LacZ construct to neurons of the CNS *in vivo*: the virus gave strong transgene expression in

striatal and nigral neurons, but no expression was observed in the cortex (Lilley et al., 2001). Characterization of neurospheres derived from different neurogenic regions showed that neural precursors maintain region specific molecular identities of their *in vivo* counterparts even after continuous passaging (Parmar et al., 2002). The presented data extend these previous findings by showing that cellular differences between neurosphere cultures define their susceptibility for HSV-1 infection. Fate differences of neural precursor cells may restrict viral attachment due to altered expression of cellular receptors, such as heparan sulphates or FGF receptors, or alternatively, cause a region-specific promoter shut off.

Limited transgene expression after transplantation in vivo

Virally transduced neurospheres expressed the reporter gene after striatal transplantation for only a short time *in vivo*. Aggregates of GFP expressing cells were only found within three days after transplantation and transduced cells either ceased transgene expression or were eliminated due to a host immune response. Since it was decided to transplant whole neurospheres that were previously shown to have better survival rates after grafting than dissociated precursor cells (Ostenfeld et al., 2000), a steel needle with large diameter (28 gauge) was used that caused major cellular damage around the injection site. This damage resulted in a significant upregulation of GFAP and nestin expression around the injection site three days post-grafting suggesting microglia infiltration. Thus, microglia cells may have eliminated the graft and could be the reason for the lack of transduced precursor cells found at later time points. Cells expressing TH were detected in close proximity to the graft and may have resulted from either primary dopaminergic present within the transplanted precursor cells or from differentiation of uncommitted precursor cells after transplantation. Transient expression of TH positive neurons within intrastriatal grafts have also been found after transplantation of human progenitors into depleted striatum (Ostenfeld et al., 2000). It has been suggested that the adult rat striatum retains at least some of the endogenous cues required to support the development of dopaminergic neurons from undifferentiated NSCs. However, no TH positive cells were found in this study at later stages which is in agreement with the reported findings (Ostenfeld

et al., 2000). As grafting was not a major aim of this study, no attempt was made to further improve the technical difficulties associated with the surgical procedure.

Disabled HSV-1 deliver transgenes to cells within the stem cell niche in situ

In the last part of this chapter it was shown that replication-deficient HSV-1 transduced cells of the SVZ-ependymal region, a region that has well been established to harbour neural stem cells giving rise to new neurons within the adult mammalian brain (Alvarez-Buylla et al., 2001; Alvarez-Buylla and Garcia-Verdugo, 2002; Alvarez-Buylla and Lim, 2004). Although an identification of neural stem cells within this region is difficult due to the lack of appropriate unique stem cell markers, it was demonstrated that HSV-1 can deliver recombinant genes to this microenvironment that can best be described as "stem cell niche" (Alvarez-Buylla and Lim, 2004; Fuchs et al., 2004). Ependymal and subependymal cell layers of the ventricle wall were transduced and can be used to deliver genes into the close neighbourhood of the actual stem cells. Hence, secreted factors can be applied to modify the stem cell niche in order to study proliferation or differentiation of endogenous precursor cells since "...'stemness' may be more related to competence of a group of early precursors within a lineage rather than to a specific cell type of cell" (Alvarez-Buylla and Lim, 2004). Viral transduction of precursor cells within the SVZ did not inhibit their ability to migrate to their final destination, such as the olfactory bulb. Since these cells have been shown to divide during their migration (Lois and Alvarez-Buylla, 1994), cell division apparently did not affect the functional integrity of the viral genome. Similar results were shown for subependymal injections of adenoviral vectors, followed by stable expression of reporter genes in differentiated granular and periglomerular neurons in the olfactory bulb (Yoon et al., 1996). Viral leakage or retrograde transport were ruled out as these neurons do not project outside the olfactory bulb (Yoon et al., 1996).

Characterization of the antigenic character of the transduced precursor cells within the SVZ showed no cell type specific reporter gene expression. Due to the use of an unspecific CMV promoter, reporter gene expression was observed in GFAP positive astrocytes and in nestin positive precursor cells. Some of the GFAP/GFP double

labelled cells showed characteristic cell morphologies of type B astrocytes that have been demonstrated to give rise to fast dividing precursor cells (type C cells) (Doetsch et al., 1999).

This chapter has shown that disabled HSV-1 transduces neural progenitor cells maintained as monolayer, neural stem cells expanded as neurosphere cultures and endogenous neural precursor cell. The optimal vector for each of these cell systems can be considered as function of gene delivery efficiency and adverse effects from viral transduction. For efficient delivery to progenitor cells at early time points, the less disabled backbone RL1+/27+/4- is the vector of choice. This is also the case for cortical rodent and human neurospheres that are otherwise only difficult to transduce with the highly disabled vector. However, this 1764/27-/4- based vector efficiently transduces striatal and mesencephalic neurospheres without any evident cytotoxic effects. Although the less disabled vector also delivers to these cells at high efficiency this is achieved on expense of neuronal cell loss most likely due to cytotoxic effects. No obvious differences between the two tested viral vectors were observed *in vivo* making both vectors equally useful for delivery to endogenous precursor cells.

CHAPTER 4:

FGF8B IS A MITOGEN FOR NEURAL PRECURSOR CELLS *IN VITRO*

4.1 Introduction

A plethora of growth factors have been studied regarding their effects on the proliferation and differentiation of neural precursor cells *in vitro* and *in vivo* (Kilpatrick and Bartlett, 1993; Ahmed et al., 1995; Caldwell and Svendsen, 1998; Erlandsson et al., 2001; Gallo et al., 2002; Farkas et al., 2003). Fibroblast growth factors (FGFs), a family of 23 known members, play a central role in nervous system development (Vaccarino et al., 1999; Ford-Perriss et al., 2001; Ornitz and Itoh, 2001) and modulate a variety of biological activities including proliferation, migration and survival of neurons and glia (Vaccarino et al., 1999; Ford-Perriss et al., 2001). The function of FGF2 on the proliferation of embryonic precursor cells has been well established (Baird, 1994), but initial studies identified EGF as the first mitogen for the expansion of NSC's isolated from adult tissue (Reynolds and Weiss, 1992; Vescovi et al., 1993). Later it has been shown that the forebrain germinal zone and its adult remnant, the subependyma, contain a small population of EGF and FGF2 responsive stem cells (Vescovi et al., 1993; Morshead et al., 1994; Palmer et al., 1995; Gritti et al., 1996), which display the definite characteristics of stem cells, i.e. self-renewal and multipotentiality. Indeed it has been shown that E14 striatal neural precursor cells initially respond only to FGF2 and acquire EGF responsiveness later during *in vitro* development (Ciccolini and Svendsen, 1998). This transition in growth factor responsiveness was also demonstrated to occur during CNS development *in vivo* (Tropepe et al., 1997; Ciccolini, 2001). EGF responsive precursor cells resulted from symmetric division and asymmetric division of FGF2 responsive stem cells (Martens et al., 2000). EGF and FGF2 are the most commonly used growth factors for the expansion of NSC's as neurosphere cultures to date (for reviews see Weiss et al., 1996b; McKay, 1997; Gage, 2000; Doetsch, 2003), and neurospheres generated with these mitogens retain the ability to produce neurons, astrocytes and oligodendrocytes conferring on them their stem cell identity (Reynolds et al., 1992; Weiss et al., 1996a).

Recently other factors have also been identified which provide a mitogenic potential to neural precursor cells *in vitro*. It has been shown that sonic hedgehog (Shh) induces proliferation of adult hippocampal precursor cells (Lai et al., 2002). However, the

study did not address if the morphogen also has an effect on ventricular derived stem cells. PDGF has mitogenic effects on the expansion of immature neuronal progenitors (Erlandsson et al., 2001) and amphiregulin, an EGF related growth factor, can be used to grow adult neural stem cells derived from the SVZ or hippocampus as neurospheres without losing their multipotent character (Falk and Frisen, 2002).

Many of these growth factors have also been demonstrated to induce proliferation of endogenous neural stem cells. EGF injected into the lateral ventricle increased the proliferation of subependymal cells. However, most of the precursor cells generated differentiated into astrocytes (Craig et al., 1996; Kuhn et al., 1997) and it was later demonstrated that EGF actually acts on rapidly dividing transit-amplifying C cells (Doetsch et al., 2002). FGF2 delivered to the lateral ventricles also increased proliferation of endogenous precursor cells but resulted in an increased number of newly generated neurons migrating to the olfactory bulb (Kuhn et al., 1997). EGF and FGF2 delivered to the fourth ventricle only generated astrocytes and oligodendrocytes (Martens et al., 2002). Intrastriatal administration of TGFα induced a massive proliferation, migration and differentiation of neural precursor cells in animals that were lesioned with 6-hydroxydopamine in the substantia nigra (Fallon et al., 2000), whilst TGFα knockouts have decreased SVZ proliferation and fewer neurons reaching the olfactory bulb (Tropepe et al., 1997). Overexpression of Shh in the SGZ induces proliferation and neurogenesis of hippocampal progenitors *in vivo* (Lai et al., 2002), while removal of the co-receptor for Shh reduced the number of progenitors in the postnatal subventricular zone and hippocampus (Machold et al., 2003). The notion that Shh may be a stem cell factor that functions during embryonic development and also continues to play a role in the "maintenance" of the adult stem cell niche is intriguing. On the other hand, increased numbers of dividing cells in the SVZ contributing to more neuroblasts migrating to the olfactory bulb may also be due to survival effects as shown after intraventricular delivery of BDNF (Zigova et al., 1998; Benraiss et al., 2001).

A member of the FGF family, FGF8b, plays a crucial role in forebrain (Storm et al., 2003) and midbrain (Danielian and McMahon, 1996) development where it is particularly required for early commitment of dopaminergic neurons (Ye et al., 1998).

FGFs exert their activity by activating a class of transmembrane tyrosine kinase FGF receptors (FGFRs) of which neural precursor cells express FGFR1 and FGFR3 (Hajihosseini and Dickson, 1999; Lobo et al., 2003). While FGF2 transduces exclusively via FGFR1 (Brickman et al., 1995), FGF8 acts mainly through the FGFR3 IIIc splice variant (MacArthur et al., 1995; Ornitz et al., 1996) that is highly expressed in the ventricular zone (Fu et al., 2003). Blocking of FGF8 signalling by soluble FGFR3 IIIc antibodies inhibits dopamine neuron differentiation (Ye et al., 1998).

Using retrovirally tagged clonal analysis it was demonstrated that FGF8b has survival effects on primary cortical precursor cells, and that most of the generated clones acquired an astroglial cell fate (Hajihosseini and Dickson, 1999). Mesencephalic precursor cells maintained in lowered oxygen show a strong upregulation of FGF8 expression accompanied by an increase in the number of dopaminergic neurons and addition of exogenous FGF8 prolonged precursor proliferation while it delayed neuronal differentiation (Studer et al., 2000). Ectopic expression of FGF8 in the mesencephalon continued neural precursor proliferation and prevented neurogenesis (Lee et al., 1997). However, previous studies indicating potential proliferative effects of FGF8 on precursor cells have not considered if FGF8b may be sufficient as the only source for the continuous expansion and passaging of neural stem cells.

The following chapter considered if foetal neural stem cells derived from different neurogenic regions can be expanded as neurosphere cultures with FGF8b as the only growth factor without losing stem cell identity. With respect to its role on defining dopaminergic phenotypes during development, possible inductive effects of FGF8 on *de novo* generation of dopaminergic neurons from mesencephalic precursor cells was also examined.

4.2 Materials and Methods

4.2.1 Determination of primary neurospheres formed

The striatum (medial and lateral ganglionic eminence) was dissected from E14 Sprague-Dawley rats and dissociated into a single cell suspension as described in 2.3.6. Cells were seeded at 20 viable cells/μl into Poly-HEMA coated (2.3.11) 24 multiwell dishes in a total volume of 1ml DMEM/F12/B27 medium containing heparin (5μg/ml) and either FGF8b (50ng/ml), FGF2 (20ng/ml) or BSA (20ng/ml). Three days after seeding 1ml fresh medium with recombinant protein was added per well. On 5 DIV the total number of formed neurospheres (>10cells per aggregate) were counted from six different wells. The means were calculated from three independent experiments.

4.2.2 Growth curves

E14 cortex, striatum and ventral mesencephalon were dissected as described in 2.3.6 and seeded as single cell suspension at clonal density (20 viable cells/μl) in DMEM/F12/B27 medium containing heparin (5μg/ml) and either FGF8b (50ng/ml) or FGF2 (20ng/ml). After 10 days expansion the neurospheres were collected and washed three times in DMEM/F12/B27 medium to remove traces of growth factors and heparin. For each region and growth factor condition 12 medium sized neurospheres were selected (about 120μm diameter) and single spheres were transferred into each well of a Poly-HEMA coated 96 well plate containing proliferation media with FGF8 (50ng/ml) or FGF2 (20ng/ml) as solely mitogen either in the absence or presence of heparin (5μg/ml). The diameter of each neurosphere was measured from two different angles under a standard light microscope with a reticulate ocular and the mean of the diameters calculated. Based on the assumption that neurospheres had the shape of a perfect sphere, the volume was calculated for each treatment every three days in a total period of 12 days.

4.2.3 Dose response curves

Dissociated striatal E14 precursor cells were seeded at clonal density (20 viable cells/μl) in T75 tissue culture flasks (Nunclon) containing 5ml of DMEM/F12/B27 medium with heparin (5μg/ml) and the growth factors FGF2 or FGF8 at a final concentration of 0.1ng/ml, 1ng/ml, 10ng/ml and 100ng/ml, respectively. Three days after plating 5ml fresh medium was added per treatment. On 6 DIV each culture was pulsed by adding BrdU to a final concentration of 0.2μM. 14 hours later the neurospheres were collected dissociated into a single cell suspension as described in 2.3.9 and plated at a density of $1x10^5$ cells per well on poly-L-lysine/Laminin coated glass coverslips (2.3.10). One hour later cells had attached to the surface and were fixed with 4% paraformaldehyde. Immunocytochemical staining for BrdU was performed as described in 2.5.2.2 and cells were counterstained with DAPI to label cell nuclei. The percentage of BrdU labelled cells of the total cell number was determined from 10 different fields.

4.2.4 Characterization of FGF8 expanded neurospheres

E14 cortical, striatal and mesencephalic precursor cells were seeded at clonal density (20 viable cells/μl) in media containing heparin (5μ/ml) and either FGF8 (50ng/ml) or FGF2 (20ng/ml). After 10 days expansion cells were dissociated into a single cell suspension (2.3.9) and plated at a cell density of $5x10^4$ cells per well on poly-L-lysine/Laminin coated glas cover slips (2.3.10). Cells were differentiated in the absence of exogenous growth factors for four days following fixation with 4% paraformaldehyde (w/v in PBS). Immunocytochemistry for TuJ1, GFAP and GalC were performed as described in 2.5.2.2 and cell nuclei were counterstained with Hoechst. The number of astrocytes, neurons and oligodendrocytes was determined from 10 randomly chosen fields and the mean calculated from three independent experiments.

4.2.5 Sectioning of E12 ventral mesencephalic tissue

Ventral mesencephalon of time mated E12 Sprague-Dawley rats were dissected as described in 2.3.6. E12 explants were mechanically dissociated by chopping into 300μm sections using a tissue-chopper as described for passaging of human neurospheres in 3.2.3. Cells were expanded in DMEM/F12/B27 media containing heparin (5μg/ml) and as indicated FGF8 (50 ng/ml), FGF2 (20ng/ml), ShhN (50ng/ml) or BSA (20ng/ml). At the indicated time points neurospheres were passaged by chopping into 200μm sections as described before. For differentiation neurospheres were dissociated into a single cell suspension (2.3.9) and plated at a cell density of $5x10^4$ cells per well on poly-L-lysine/Laminin coated glass coverslips (2.3.10). During differentiation cells were maintained in DMEM/F12/B27 media in the absence of any growth factors. After 4 days cells were fixed with 4% paraformaldehyde (w/v in PBS) and immunocytochemical staining performed as described in 2.5.2.2.

4.2.6 RT-PCR analysis

Rat E12 ventral mesencephalon and cortex was dissected as described in 2.3.6 and mechanically dissociated by chopping into 300μm sections. Explants were expanded as neurosphere cultures with combinations of ShhN (100ng/ml), FGF8 (200ng/ml), FGF2 (20ng/ml) or BSA (200ng/ml) in Poly-HEMA coated 12 well plates (2.3.11) as illustrated in Figure 4-10. Neurospheres were passaged by mechanical dissociation into 200μm sections on 2 DIV, 10DIV, 14DIV and 21DIV. After 12DIV and 30DIV aliquots were taken for RNA preparation and differentiation by growth factor withdrawal for four days. RNA was extracted as described in 2.2.12, DNAse treated (2.2.13) and first strand cDNA synthesis was performed from 10μg RNA per condition using the Superscript kit (Invitrogen) (2.2.14). cDNA was treated with 1μl RNAse H (Invitrogen) per reaction for 20min at 37°C. PCR conditions were optimized by varying $MgCl_2$ concentration, annealing temperature and cycle number to determine linear amplification rate. $MgCl_2$ concentration was 2.0mM for TH and 1.5mM for all others. PCR was performed with Platinum Taq DNA Polymerase (Invitrogen) at the following program: 94°C for 2 min, cycles with denaturation at

94°C for 15 sec, annealing for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 10 min. Primer sequences, cycle numbers and annealing temperatures were as follows:

GAPDH (833-1137): 28 cycles, 59°C, 305 bp
FW: 5' CTCGTCTCATAGACAAGATGGTGAAG
RW: 5' AGACTCCACGACATACTCAGCACC

Nurr1 (194-448): 30 cycles, 55°C, 255 bp
FW: 5'TGAAGAGAGCGGAGAAGGAGATC
RW: 5'TCTGGAGTTAAGAAATCGGAGCTG

Ptx3 (378-634): 35 cycles, 60°C, 257 bp with 5% DMSO
FW: 5' CGTGCGGGTGTGG TTCAAGAAC
RW: 5' GCGGTGAGAATACAGGTTGTGAAG

En-1 (1164-1544): 30 cycles, 60°C, 381 bp;
FW: 5'TCAAGACTGACTCACAGCAACCCC
RW: 5'CTTTGTCCTGAACCGTGGTGGTAG

TH (1130-1428): 27cycles, 56°C, 300bp
FW: 5' CACTGTGGAATTCGGGCTATG
RW: 5' GGCTGTCCAGTACGTCAATG

PCR products were analysed on 1-2% agarose gels containing ethidium bromide (1μg/ml).

4.2.7 Proliferation of endogenous neural stem cells

Intraventricular injections were performed as described in 3.2.5 using $5x10^6$ PFU of RL1+/27+/4-pR19FGF8bwpre, RL1+/27+/4-pR19LacZwpre or 1764/27-/4-/pR19FGF2 per injection and delivered into both ventricles of adult female Sprague-Dawley rats (n=4 per group) (260-280g). The rats were injected daily with the mitotic

marker bromodeoxyuridine (BrdU) (100mg/kg, i.p.) for a total period of 18 consequtive days. The animals were perfused with 4% PFA two days after the last BrdU injection (day 20). The brains were removed including olfactory bulbs, post-fixed for 1h in 4%PFA and protected in 30% sucrose. After the brains had sunk they were cut as 20μm sagittal (n=3) or coronal (n=1) sections on a freezing microtome. Immunohistochemistry for BrdU, NeuN, GFAP, nestin, TH was performed as described in 2.5.3.

4.3 Results

In preliminary work it appeared that neurospheres expanded with FGF2 in the presence of FGF8 proliferated much faster than in the absence of FGF8 or in the presence of other morphogens such as sonic hedgehog. This prompted the idea that FGF8 itself may be a mitogen for neurospheres that could be used independently of other growth factors for the expansion of neural precursor cells.

4.3.1 Neural stem cells expanded in FGF8 retain their neural stem cell identity

To test if NSC's can be proliferated in FGF8 as the only mitogen, precursor cells isolated from rat E14 cortex, striatum and ventral mesencephalon were seeded at clonal density (20 viable cells/μl) in DMEM/F12 media containing heparin (5μg/ml) and recombinant mouse FGF8b (50ng/ml, R&D). Control cultures contained FGF2 (20ng/ml) or no exogenous growth factors. The NSC's were grown in suspension culture to obtain neurospheres that could subsequently be passaged. The cells seeded as a single cell suspension started forming multicellular clusters after one to two days in culture. Significantly more clusters were observed in growth factor containing cultures compared to cultures without exogenously added mitogens. The cells continued dividing rapidly and after about five days neurospheres were clearly visible in FGF8 and FGF2 containing cultures. Differences in the proliferation rate depending on the mitogen were evident (see below). FGF2 containing cultures formed larger neurospheres than those in the presence of FGF8 despite the lower concentration of FGF2. However, morphologically the neurospheres were indistinguishable (for pictures of FGF8 and FGF2 expanded neurospheres see Figure 4-5). FGF8 expanded neurospheres comprised a dense cluster of cells and the surface of the spheres appeared smooth with some cells in the outer layer projecting short processes into the medium. This morphology is identical to FGF2 expanded neurospheres and is considered the "healthy" appearance for a neurosphere. Formations of spherical aggregates under mitogen free conditions were also found. However, these neurospheres were much smaller and appeared as rather loose cell association without any visible cilia. After dissociation these spheres did not form any secondary spheres.

They were considered as aggregates formed from residual cell division of mitotic active primary neural precursor cells or due to endogenous release of growth factors stimulating short term cell division.

After 10 days, mitogen containing cultures had formed large neurospheres that were dissociated into single cells and seeded under the same culture conditions as before. Secondary neurospheres appeared at about two days after dissociation from FGF2 and FGF8 containing cultures, but not from growth factor free controls. After one week these secondary spheres were dissociated again to seed cultures forming tertiary neurospheres that were plated onto poly-L-lysine/laminin coated glass coverslips. The neurospheres were differentiated by withdrawal of any exogenous growth factors and as shown in the previous chapter, cells started forming radial processes and migrated into the substrate. Process formation and migration of neural precursor cells was observed independent of the mitogen used for expansion. After four days of cell differentiation, cells were stained for markers identifying the three neural lineages: TuJ as neuronal marker, GFAP as marker for astrocytes and GalC for oligodendrocytes (Figure 4-1). Independent of the neurogenic region FGF8 expanded neurospheres generated neuronal, glial and oligodendroglial progenitor cells. Hence, it was concluded that FGF8 may be a mitogen for embryonic neural precursor cells that retain their stem cell identity even after subsequent passaging.

Neurospheres could be continuously expanded and passaged in FGF8 as the only available exogenous mitogen for a period of about four to five weeks without losing their multipotent character. After this period cells had significantly reduced growth rates and underwent senescence as has been shown for EGF and FGF2 expanded rodent neural stem cells (Svendsen et al., 1997b).

Effects of FGF8 on the formation of primary cell clones were determined for striatal precursor cells seeded at clonal density (Figure 4-2). Although in the presence of FGF8 slightly more primary neurospheres were generated than in FGF2, the differences were not statistically significant. In BSA alone a significantly smaller number of very small neurospheres were generated showing the growth of neurospheres is not favoured in the absence of exogenous mitogens.

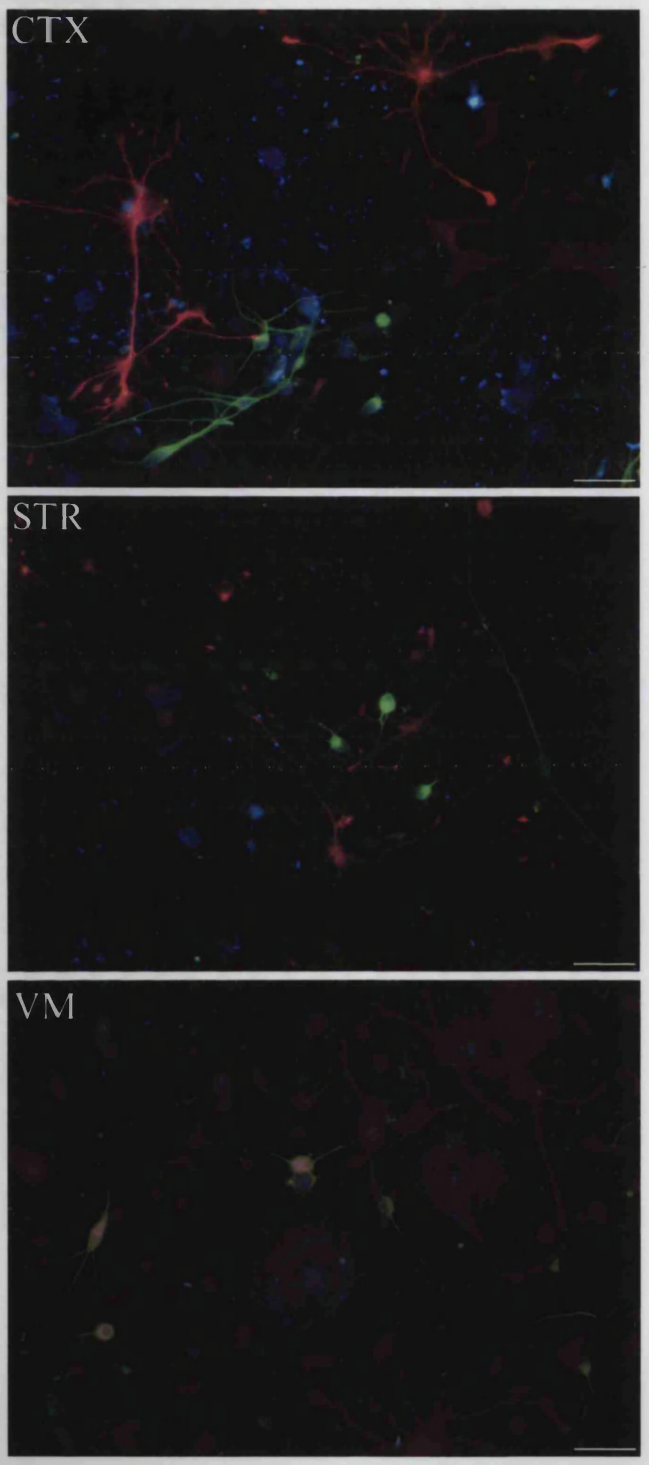


Figure 4-1: FGF8 expanded neurospheres retain the capacity to differentiate into the three neural lineages after multiple passaging. Neural precursor cells derived from E14 cortex (CTX), striatum (STR) and ventral mesencephalon (VM) were expanded as neurosphere cultures in medium (DMEM/F12) containing heparin (5µg/ml) and recombinant mouse FGF8b (50ng/ml, R&D) but in the absence of any other growth factors. Neurospheres were passaged twice by dissociation into a single cell suspension and seeding at clonal density (20 viable cells/µl) prior to plating and differentiation in the absence of FGF8b. Shown is the triple immunolabelling for the neuronal marker TuJ (Alexa 488, green), the astrocytic marker GFAP (Alexa 546, red) and the oligodendroglial marker GalC (Alexa 350, blue). Scale bar represents 50µm.

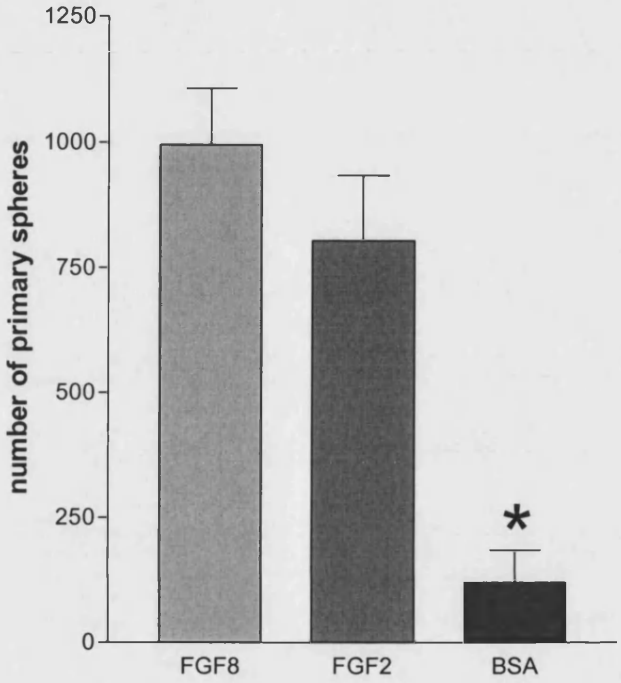


Figure 4-2: Number of primary formed neurospheres is unaltered between FGF8 and FGF2. E14 striatum tissue was dissociated into a single cell suspension and cells seeded at clonal density (20viable cells/ml) into a 24well plate. Cells were expanded in DMEM/F12 media with heparin (5μg/ml) containing either FGF8b (50ng/ml), FGF2 (20ng/ml) or BSA (20ng/ml), respectively. The total number of primary neurospheres per well was counted after 5 DIV from 6 wells per treatment. Data illustrated represent the means of total neurospheres per well with SEM from three independent experiments. No statistical significant differences in the number of primary neurospheres were determined between FGF8 vs. FGF2 ($p>0.05$ in One-way ANOVA, n=3 experiments). However, striatal precursor cells maintained in BSA as control formed significantly less neurospheres ($p<0.05$ in One-way ANOVA, n=3 experiments).

4.3.2 Heparin increases the mitogenic effects of FGF8

As discussed above, although the number of neurospheres generated from clones was unaltered between the use of FGF8 or FGF2, they appeared to grow much faster in the presence of FGF2. The previous chapter discussed, that heparin sulphates plays a crucial role in FGF signaling and the mitogenic effects of FGF2 on the expansion of particularly mesencephalic precursor cells (Caldwell and Svendsen, 1998). Heparin and heparin sulphate domains also play an important role in binding and signaling of FGF8b (Loo and Salmivirta, 2002) but their function on the proliferation of neural precursor cells induced by FGF8 has not been reported. Here, growth curves were performed for neural precursors expanded in FGF8 either in the presence or absence of heparin and the proliferative effects of FGF8 compared to those of FGF2.

Heparin significantly increased the mitogenic activity of FGF8 for the three neurogenic regions (cortex, striatum and ventral mesencephalon) (Figure 4-3). In the absence of heparin neural precursor cells grew very slowly with a 3-fold increase in the sphere volume after 12 days. No significant differences in the growth rates between the different regions were observed under these conditions. However, adding heparin to the medium considerably changed the growth pattern. Region specific differences were observed in the proliferation rates showing that striatal (12 days $p<0.001$, n=12 neurospheres) and cortical (12 days $p<0.01$, n=12 neurospheres) derived neurospheres proliferated significantly faster when compared to mesencephalic, while no significant differences were observed between striatal versus cortical NS (12 days $p>0.05$, n=12 neurospheres) under these conditions. In the presence of heparin mesencephalic neurospheres underwent an approximate 10-volume increase, cortical neurospheres a 20-fold increase and striatal neurospheres a more than 30-fold increase in the sphere volume after 12 days expansion. We conclude that, as for FGF2, supplementation of exogenous heparin is required for the mitogenic activity of FGF8 on neural precursor cells grown as neurospheres.

Comparing the mitogenic effects of FGF8 to those of FGF2 (Figure 4-4) showed a similar pattern for the expansion of NSC's from different regions with either growth

factor: mesencephalic neurospheres grew slower than those derived from the forebrain, an observation that conforms to other reports (Ostenfeld et al., 2002a). Furthermore, the growth curves showed that FGF2 was a more potent mitogen for the expansion of cortical and striatal neurospheres than FGF8. In the presence of heparin an 80-fold increase in the sphere volume was calculated for ^{ctx}NS after 12 days in culture and for ^{str}NS this increase was 100-fold. Post hoc comparisons with FGF8 expanded neurospheres revealed significant differences ($p<0.001$ at 12 days, n=12 neurospheres) for ^{ctx}NS and ^{str}NS, however, ^{mes}NS did not reveal increased proliferation rates in FGF2 compared to FGF8 ($p>0.05$ at 12 days for n=12 neurospheres). This might reflect a relatively higher affinity of mesencephalic neural precursors to the mitogenic effects of FGF8 than those of FGF2.

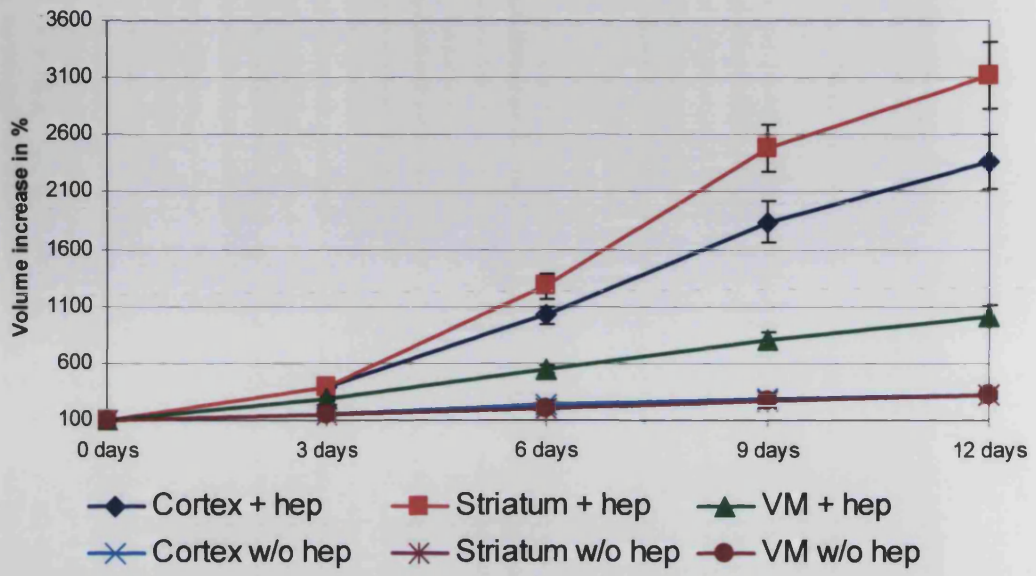


Figure 4-3: Heparin significantly increases the mitogenic effects of FGF8. E14 derived NS were expanded for 10 days before medium sized neurospheres were selected. Diameters of 12 neurospheres were measured at the indicated time points in three independent experiments. One way ANOVA revealed significant differences in the growth rate of VM vs. STR/CTX ($p<0.01$; n=12 neurospheres) and significant increase in the presence of heparin ($p<0.0001$ for all). No differences were detected for the different cell lines grown in the absence of heparin.

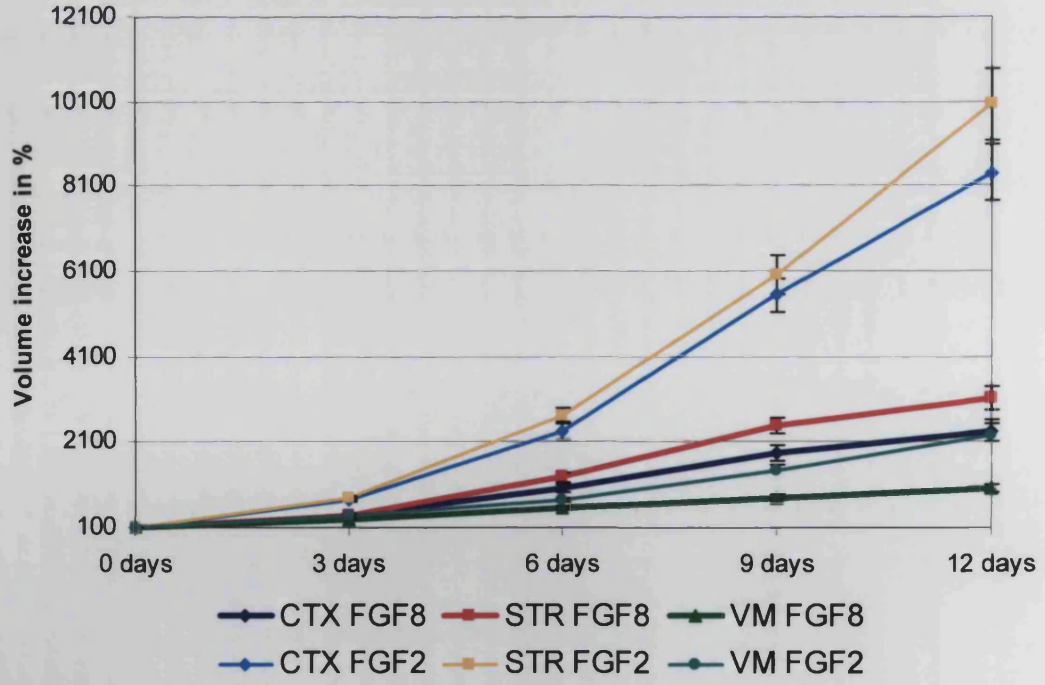


Figure 4-4: FGF8 expanded NS grow significantly slower than in FGF2. Neurospheres from the different neurogenic regions were expanded either in the presence of FGF8b (50ng/ml) or FGF2 (20ng/ml). Medium sized NS were selected and the diameter measured over the indicated time period. One-way ANOVA with Tukey's post hoc test shows significant differences in the growth rate for CTX and STR NSC's expanded in FGF8 vs. FGF2 ($p<0.001$; n=12 neurospheres), but not for VM NSC's.

4.3.3 FGF8 dose response curve for ^{str}NS

To further confirm differences in the affinity for the two mitogens, dose response curves were carried out on striatal neural precursor cells for FGF8 and FGF2 (Figure 4-5).

For both mitogens a sigmoid correlation between the number of BrdU incorporating cells and the growth factor concentration was found. However, at the same mitogen concentration more cells showed a mitotic response to FGF2 compared to FGF8 suggesting higher proliferation rate of the precursor cells for FGF2. While at high FGF2 concentrations (100ng/ml) about 90% of the cells were mitotic active, the same concentration of FGF8 stimulated cell division in only 67% of the striatal precursor cells. Although saturating mitogen concentration were not tested, the data suggest that the concentration of FGF8 required to stimulate 50% of the striatal precursors to divide is approximately about 60ng/ml, while it seems to be less than 10ng/ml for FGF2.

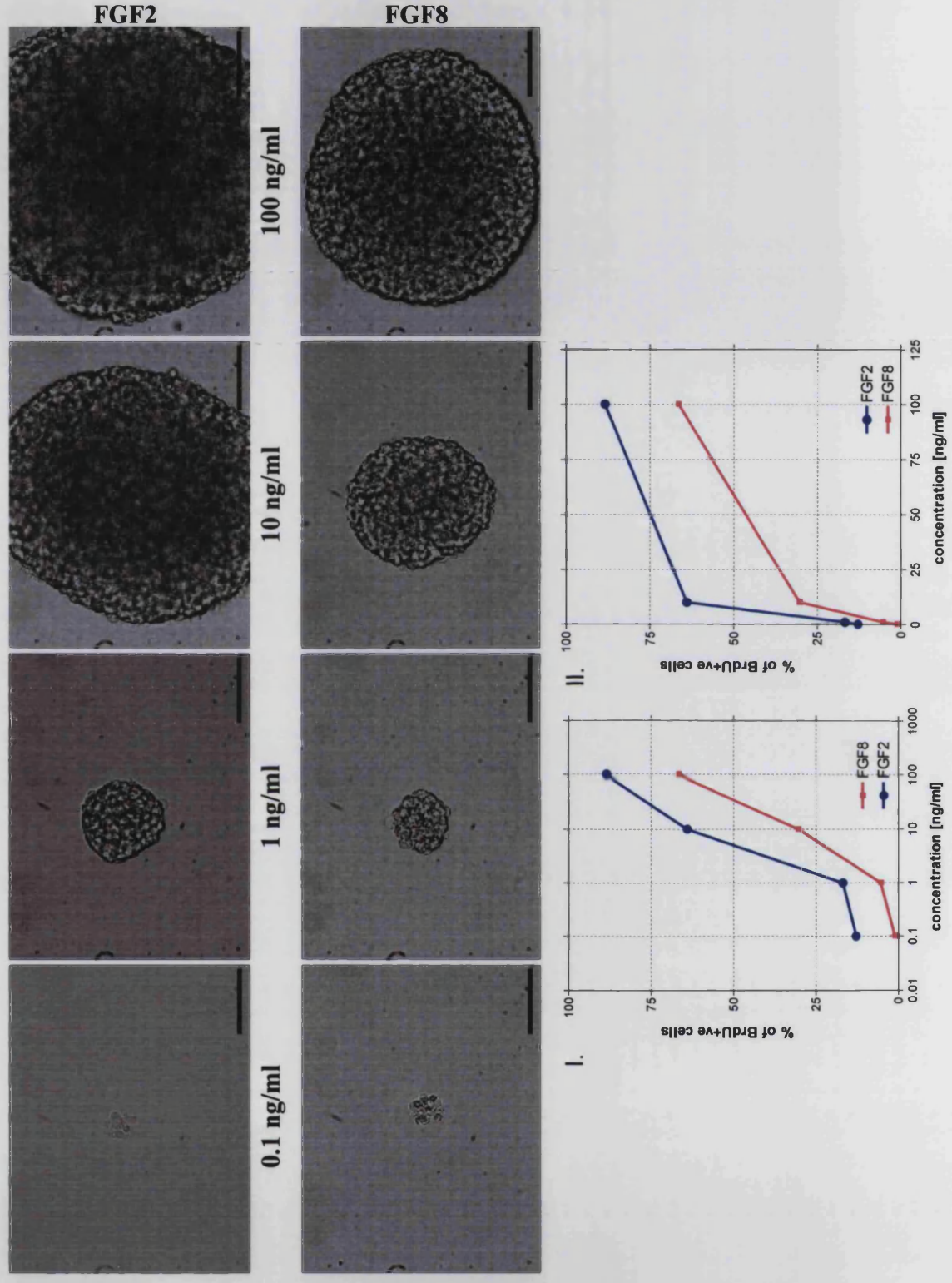


Figure 4-5: Dose response curves for FGF8. E14 striatal precursor cells were seeded at clonal density (20 cells/µl) in medium containing heparin (5µg/ml) and the indicated final concentration of either FGF8b or FGF2 as solely mitogen. On 6 DIV cultures were pulsed with BrdU (0.2µM final concentration) and neurospheres dissociated 14 hours later and plated on PLL/Laminin coated glass coverslips. Cells were fixed after attachment and stained for BrdU. BrdU positive cells as a percentage of total cell number were determined and are presented as the mean with SEM from three independent experiments. I. Sigmoid correlation between BrdU incorporation and mitogen concentration using a logarithmic scale. II. A linear scale illustrates a higher concentration is required for FGF8 to induce proliferation in 50% of the striatal precursor cells. Scale bar represents 100µm.

4.3.4 Characterization of FGF8 expanded neural precursor cells

Multipotent precursor cells derived from embryonic tissue respond differently to EGF and FGF2 (Ciccolini and Svendsen, 1998), and different neurogenic regions produce distinct numbers of neural progenitors (Ostenfeld et al., 2002a). Growth factor concentration can also affect the outcome of neural differentiation (Qian et al., 1997). Further, it has been suggested that FGF8 promotes astroglial differentiation of cortical precursor cells (Hajihosseini and Dickson, 1999). In order to study if FGF8 expanded neurospheres give rise to a different population of progenitor cells than FGF2 expanded spheres, the composition of forebrain and midbrain derived progenitor cells was characterized after differentiation (Figure 4-6).

^{ctx}NS, ^{str}NS and ^{mes}NS were expanded for one week in either FGF8 or FGF2 and following dissociation the precursor cells were differentiated by growth factor withdrawal and exposure to a substrate. Neurons, astrocytes and oligodendrocytes were identified by immunocytochemical staining with cell-type specific markers TuJ1, GFAP and Gal-C, respectively (Figure 4-6). No significant differences were observed in the proportion of the three neural cell types generated from neurospheres expanded in FGF8 compared to those expanded in FGF2. In all treatments a high proportion of cells that didn't label with either of the immunogenic markers was detected and most likely these cells represent a population of undifferentiated neural precursors. Residual growth factors or endogenous mitogens released by the precursor cells may, therefore, maintain some cells in an uncommitted neural fate. In neither the forebrain nor midbrain cultures was an increase in GFAP positive astrocytes or a decrease in neuronal progenitor cells observed when expanded in FGF8 versus FGF2, as has been described for cortical precursor cells (Hajihosseini and Dickson, 1999). Rather a decreased number of astrocytes was found but more oligodendrocytes appeared when expanded in FGF8. However, these differences were marginal. The discrepancy may have resulted from different experimental conditions as the referred to study considered single clones formed in the presence of FGF8 while this study characterized the total cell population of neurospheres generated at clonal density. Therefore some of the neurospheres generated may give rise exclusively to astrocytes while others contain only neuronal progenitor cells. This study did not further

distinguish between the antigenic characteristics of single neurospheres generated in FGF8 compared to those generated in FGF2.

Cortical neurospheres expanded in FGF8 and FGF2 tended to give rise to more neurons and oligodendrocytes but less astrocytes than striatal or mesencephalic neurospheres. In dissociated ^{mes}NS only an insignificant number of neurons was found that were not included in the results presented. Although Figure 4-1 showed that mesencephalic neurospheres expanded in either mitogen can give rise to neuronal progeny when plated as whole neurospheres, the experimental rationale chosen for this characterization resulted in a decreased number of surviving neurons. As dissociated neurospheres rather than a migration assay were used, the stress of enzyme treatment and trituration most likely caused an increased death of neural precursor cells. Therefore, the total number of neurons was lower than reported in other studies (Ostenfeld et al., 2002a) using cell migration assays.

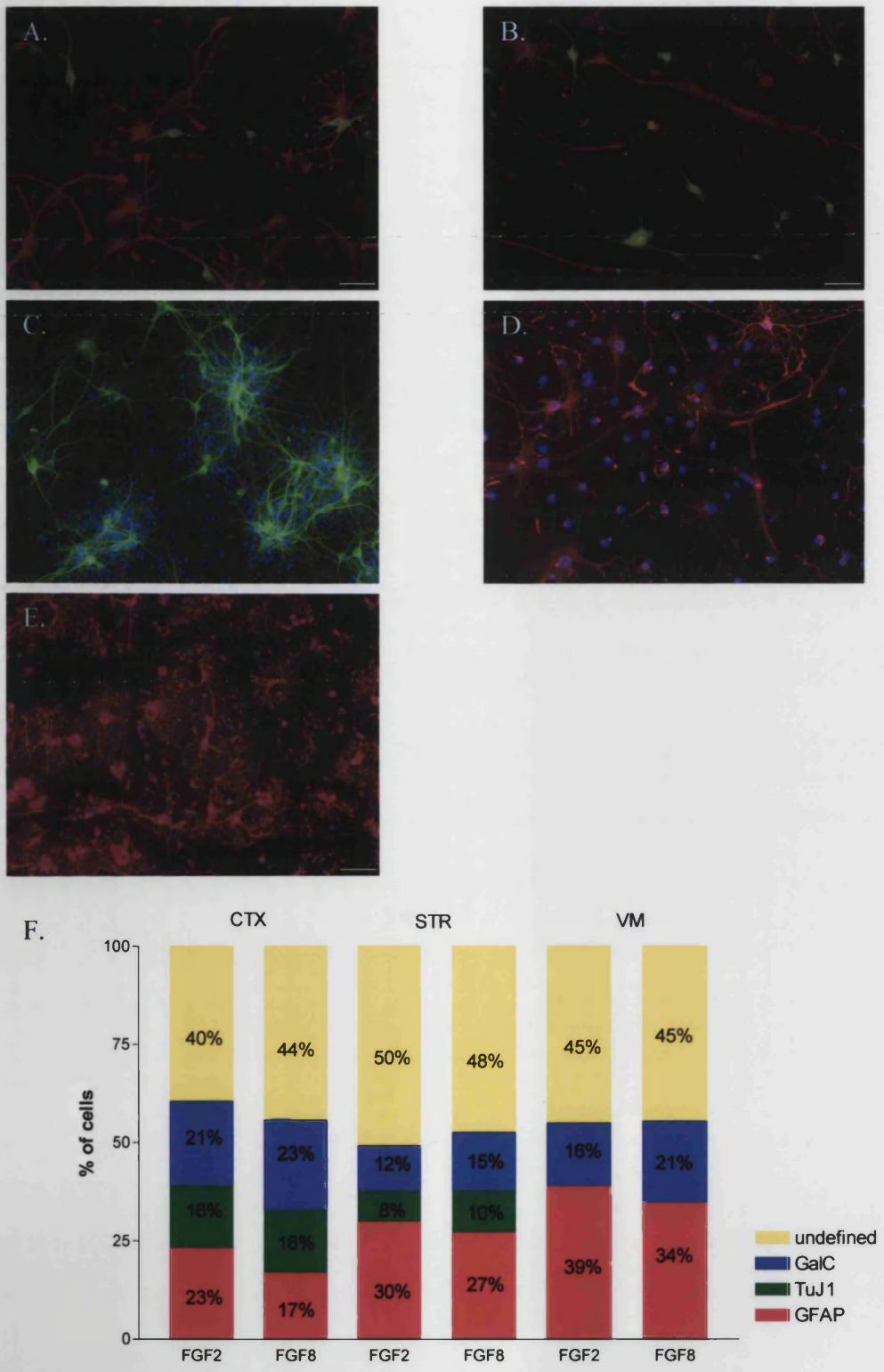


Figure 4-6: Characterization of E14 neurospheres expanded in FGF8 versus FGF2. Immunocytochemical staining for (A) FGF2 and (B) FGF8b expanded ^{ctx}NS showed for neurons (TuJ1, green) or astrocytes (GFAP, red). While FGF8 expanded ^{mes}NS contained plenty of neurons (C) prior to dissociation, after trituration (D) mainly GFAP positive astrocytes and (E) an increased number of GalC positive oligodendrocytes were observed. Cell nuclei were counterstained with Hoechst (blue). Scale bar represent 50µm. (F) Quantification showed no significant differences in the neural progeny generated when neurospheres from different neurogenic regions were either expanded in FGF2 or FGF8 as only mitogen. Data represent means as percentage of total cells from three independent experiments.

4.3.5 FGF8 increases the number of dopaminergic neurons generated from ^{mes}NS.

While FGF8 did not significantly alter the amount of TuJ1 positive neurons in neurospheres generated from cortex and striatum, it was possible that there were effects on survival or *de novo* development of dopaminergic neurons from ^{mes}NS. It is known that although FGF2 can extend dopamine neuron progenitor proliferation in E12 foetal mesencephalic cultures for a short period of time (a few days) (Bouvier and Mytilineou, 1995), further expansion results in a cell that can no longer generate DA neurons. Expansion of E14 mesencephalic precursors in FGF2 as neurospheres is possible (see previous data). However, these cultures only give rise to an insignificant number of TH positive neurons (Caldwell and Svendsen, 1998; Ostenfeld et al., 2002a), upon differentiation. Figure 4-7 shows that precursor cells generated from E14 ventral mesencephalon and seeded at clonal density as a single cell suspension gave rise to neurospheres containing TuJ1 positive neurons when expanded in either FGF2 or FGF8. However, FGF8 expanded ^{mes}NS generated a significant increase in the number of dopaminergic neurons when compared to FGF2 expanded ^{mes}NS as characterized by the expression of TH. While FGF2 expanded ^{mes}NS rarely contained TH positive cells after one week expansion, several dopaminergic neurons were detected within FGF8 expanded ^{mes}NS. After dissociation of these E14 neurospheres, using trypsin or Accutase™, the dopaminergic neurons generated did not survive as only a very few TH positive cells were detected after plating on coverslips or subsequent generation of secondary neurospheres. The decrease in TH expressing cells was accompanied by a continuous loss of total neurons with each passage as has been described for rodent neurosphere cultures (Ostenfeld et al., 2002a). Possible explanations are that enzyme based dissociation methods do not support the survival of primary dopaminergic neurons or that dopamine precursors driven by FGF8 undergo only a certain number of cell divisions and subsequently loose the ability to differentiate into dopaminergic neurons.

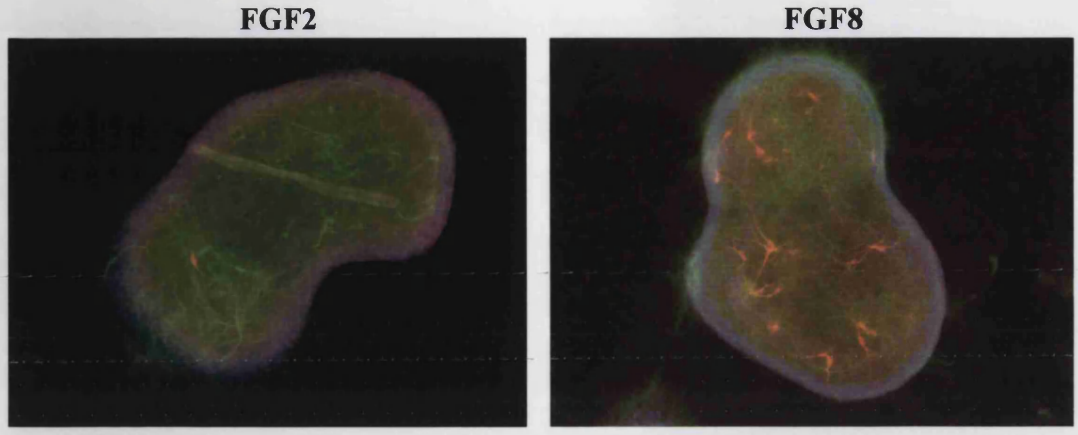


Figure 4-7: E14 ^{mes}NS give rise to more dopaminergic neurons when expanded in FGF8. Rat E14 VM was dissociated into a single cell suspension and seeded at clonal density (20 cells/µl) in either FGF2 (20ng/ml) or FGF8 (50ng/ml). The whole neurospheres were stained for TuJ1 (Alexa 488, green), TH (Alexa 546, red) and cell nuclei counterstained with Hoechst (blue). While FGF2 expanded ^{mes}NS give only rise to a very few TH positive cells, neurospheres expanded in FGF8 generated significantly more dopaminergic neurons.

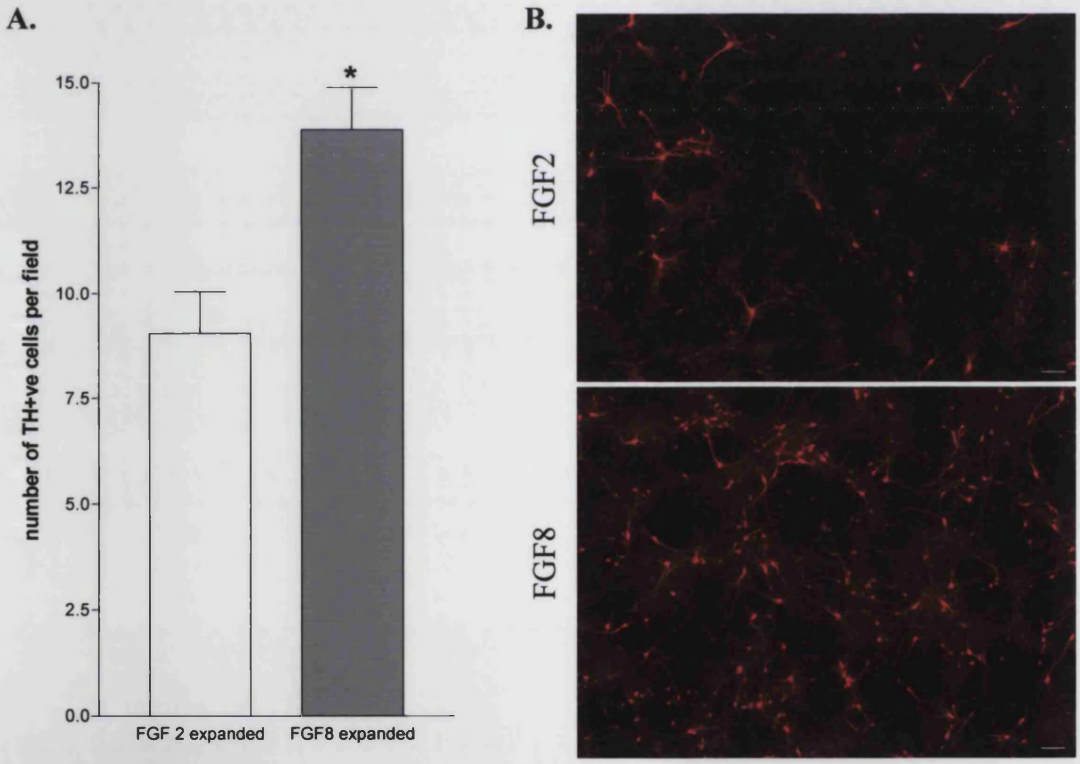


Figure 4-8: FGF8 increases the number of TH+ve cells in E12 expanded mesencephalic neurosphere culture. VM from E12 rat embryos was isolated and mechanically dissociated by chopping at 300µm. Cells were expanded for two weeks either in FGF8 (50ng/ml) or FGF2 (20ng/ml) and heparin (5ug/ml) and passaged twice by chopping at 200µm within this period. Cells were dissociated using Accutase and plated at $2x10^5$ cells on PLL/Laminin coated glass cover slides. Subsequently cells were differentiated for four days by growth factor withdrawal, fixed and stained for tyrosine hydroxylase (Alexa 546, red). A: Counting of TH+ve cells in 20 randomly chosen fields per treatment. Illustrated are means and standard error of the means (SEM). One way ANOVA with Tukey's posthoc test shows a statistically increased number of TH+ve cells when expanded with FGF8 ($p<0.01$). B: Immunocytochemistry of dissociated neurospheres previously expanded in FGF8 or FGF2 for TH (red). Image was taken at low magnification and does not represent the magnification for the countings as shown in (A). Scale bar 50 µm.

In order to quantify the differences between the number of dopaminergic neurons generated after extended proliferation in FGF8 versus FGF2 and after multiple passaging, but to avoid adverse effects of enzymatic digestion, it was decided to mechanically dissociate primary ventral mesencephalic tissue and to passage the generated neurospheres by sectioning using a tissue chopper. As discussed in the previous chapter, at E14 rat dopaminergic neurons have almost completely differentiated and thus, to increase the amount of immature dopamine neuroblasts, ventral mesencephalic tissue was dissected from time mated E12 rat embryos. E12 ^{mes}NS grew rapidly in either FGF2 or FGF8 and were passaged twice by chopping prior to dissociation with Accutase™, which was found to be less cell damaging than trypsin. Dissociated, and at equal density on poly-L-lysine/Laminin coated glass coverslips, plated precursor cells were differentiated in the absence of any growth factors and subsequently stained for TH expressing neurons.

As shown in Figure 4-8 both growth factors show an increase in the number of dopaminergic neurons compared to E14 mesencephalic tissue. However, mesencephalic precursor cells expanded in the presence of FGF8 generated statistically more dopaminergic neurons ($p<0.01$) than FGF2 cultures even after subsequent passaging. At this time point the ^{mes}NS had been in culture for two weeks and had undergone three passages in total. Consequently, the number of dopaminergic neurons can be maintained over an extended period and taken through multiple passages as neurosphere cultures using the chopping method and mesencephalic tissue from E12 rather than E14. Thus, the overall yield can be further increased with FGF8 as mitogen.

4.3.6 Sonic hedgehog further increases the number of TH positive neurons

Induction of dopaminergic neurons during neurodevelopment requires an interplay of the morphogens Shh and FGF8 *in vivo* (Ye et al., 1998). The same morphogens also play an important role for the generation of dopaminergic neurons from embryonic stem cells (Lee et al., 2000; Kim et al., 2002). However, they fail to induce a dopamine phenotype in rodent and human embryonic precursor cells expanded as neurosphere cultures with FGF2 as the mitogen (Ostenfeld et al., 1999; Svendsen et al., 1999), but seem to increase the number of TH expressing cells in a neural stem cell line overexpressing the transcription factor Nurr1 (Kim et al., 2003c). Shh and FGF8 also play a role in induction of dopaminergic phenotypes in passaged adult rat hippocampal progenitors (Sakurada et al., 1999). ShhN is neurotrophic for dopaminergic neurons and also promotes the survival of other midbrain and striatal neurons *in vitro* (Miao et al., 1997). Here, whether ShhN has effects on dopaminergic neurons generated from neural precursor cells expanded as neurospheres in FGF8 as the mitogen was explored.

E12 VM precursor cells were prepared as described in 4.3.5 and expanded as neurosphere cultures using FGF8 as the sole mitogen. After dissociation and plating as a single cell suspension on coated coverslips, cells were differentiated by FGF8 withdrawal and exposure to substrate. Recombinant ShhN (50ng/ml) was added to the neurosphere cultures either during expansion and/or the differentiation phase. As illustrated in Figure 4-9 ShhN did increase the number of TH positive dopaminergic neurons generated in FGF8 expanded cultures. Addition of ShhN during the differentiation phase did not significantly increase the number of dopaminergic neurons. However, ShhN doubled the number of dopaminergic neurons when FGF8 was simultaneously present during the expansion phase suggesting a synergistic effect of the two morphogens on dopamine neuron development from E12 mesencephalic precursor cells. By immunocytochemical staining it was confirmed that the TH expressing cells generated did co-label with the neuronal marker TuJ, and thus likely represent *bona fide* dopaminergic neurons.

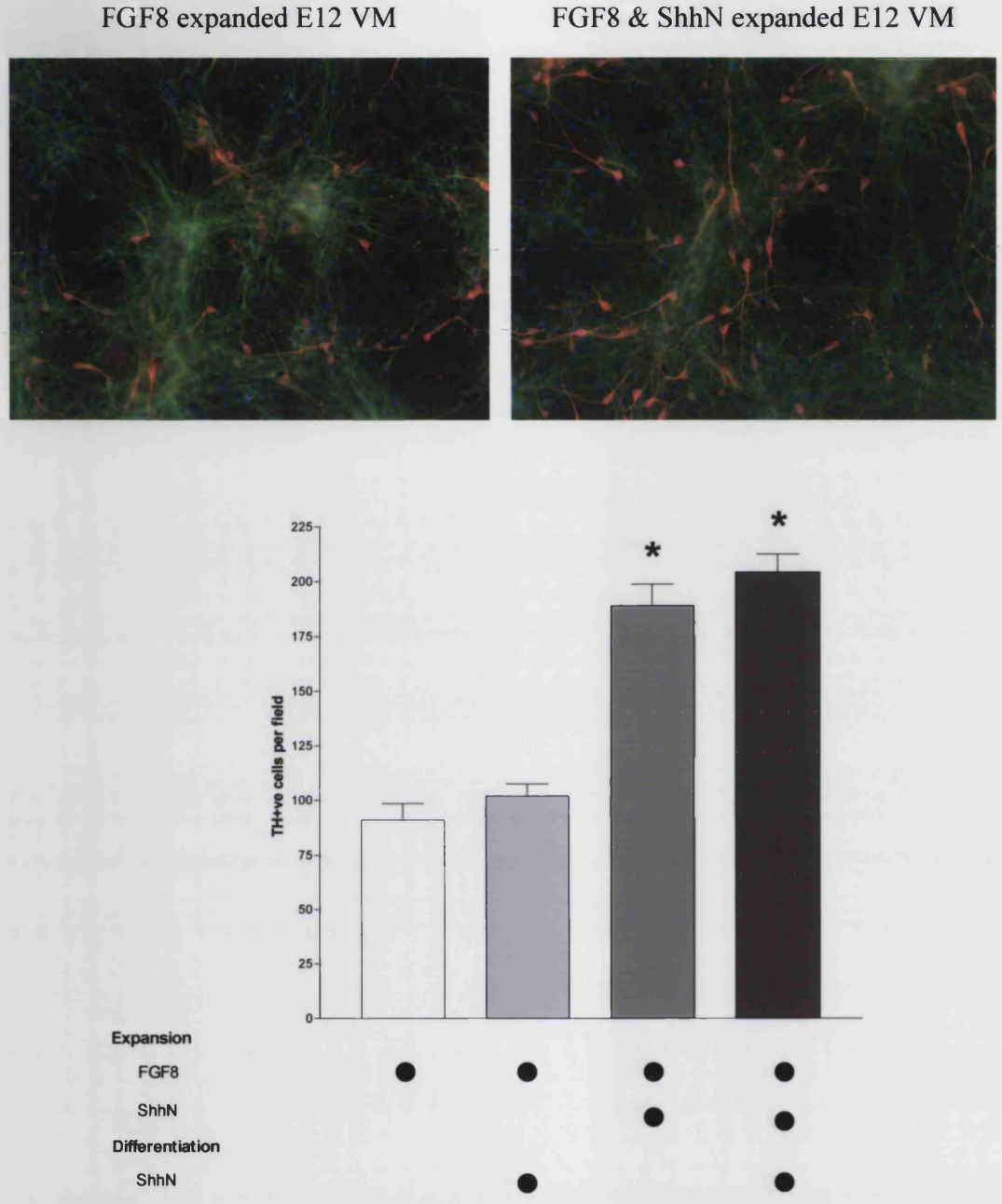


Figure 4-9: ShhN further amplifies the FGF8 mediated generation of dopaminergic neurons. E12 ventral mesencephalon was mechanically dissociated into 300μm sections. Explants were grown as neurosphere cultures in FGF8 (50ng/ml) as the mitogen in the presence or absence of ShhN (50ng/ml) during the expansion phase. After one week expansion the cells were dissociated into a single cell suspension and differentiated after plating on poly-L-lysine/Laminin coated glass cover slips for four days. During differentiation FGF8 was withdrawn but ShhN (50ng/ml) was present as indicated. Cells were fixed and stained for TuJ1 (green), TH (red) and Hoechst (blue). TH expression co-labelled with TuJ1 confirming neuronal identity. The number of dopaminergic neurons was determined from 20 different fields. One way ANOVA with Tukey's post hoc revealed a statistically increased number of TH expressing cells when ShhN was present during the expansion phase ($p<0.001$). No significant effect of ShhN was detected when the morphogen was present during differentiation phase.

To further characterize if ShhN only increases the number of dopaminergic neurons in the presence of FGF8 or if this effect was also accomplished with FGF2, mechanically dissociated E12 VM cultures were expanded in different growth factor combinations as shown in Figure 4-10. The neurospheres were passaged twice within two weeks before they were plated as a single cell suspension on laminin coated glass cover slips and allowed to differentiate for four days in the absence of mitogens. Even after passaging, FGF8 expanded neurospheres resulted in a significant increased number of dopaminergic neurons compared to when FGF2 was used as the mitogen ($p<0.001$). ShhN present during the expansion phase significantly increased the number of TH positive cells with either FGF8 ($p<0.05$ compared to FGF8 alone) or FGF2 ($p<0.01$ compared to FGF2 alone). Interestingly, when neurospheres were expanded simultaneously in both FGF2 and FGF8, either in presence or absence of ShhN, a significant drop in the number of dopaminergic neurons ($p<0.001$) was detected.

The data show that the possible inductive effect of ShhN on dopamine neuron generation from mesencephalic precursor cells is not limited to synergism with FGF8, but can also be facilitated with FGF2.

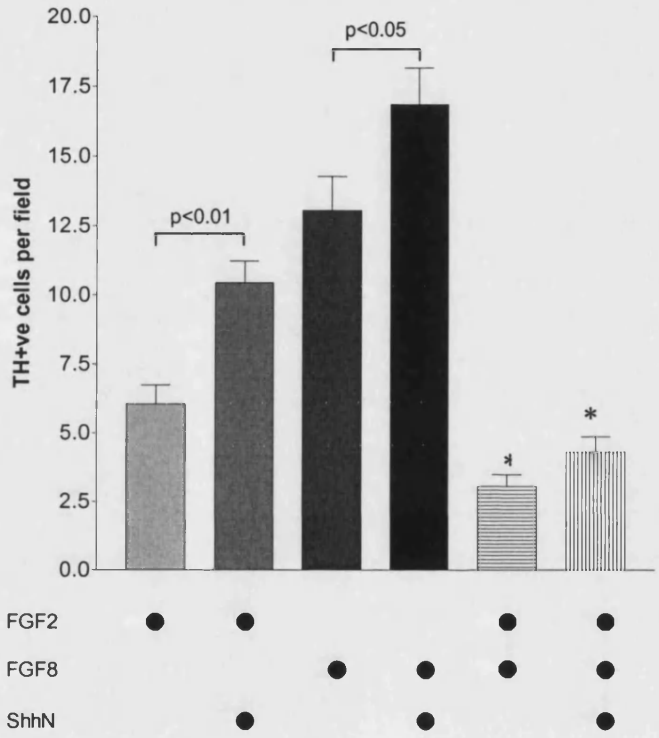


Figure 4-10: Comparison of E12 ventral mesencephalic neurosphere cultures expanded in different combinations of growth factors. Mechanically dissociated ^{mes}NS were continuously expanded by subsequent passaging in different growth factor combinations as indicated. (A) ShhN increased the number of dopaminergic neurons when present during the expansion phase, an effect that is not limited to the presence of FGF8 but was also observed with FGF2. A significantly decreased (*p<0.001) number of TH expressing cells was determined when neurospheres were expanded simultaneously in all three growth factors.

4.3.7 The expression of midbrain specific markers decreases after extended passaging

Although dopaminergic neuron containing neurospheres could be passaged over three weeks, a significant loss in the total numbers of TH expressing cells with each passage was observed in either of the growth factor combinations tested. After about four passages TH expression was detected only rarely, even during expansion with FGF8 and ShhN. If these morphogens are indeed inductive on a common multipotent precursor cell, no loss of susceptibility to the signals after consecutive passaging would be expected. Based on the idea of a unique stem cell existing in different neurogenic regions of the developing brain, an induction of dopaminergic markers from precursor cells other than those derived from the mesencephalon may be expected. To study if dopaminergic markers are upregulated in cortical neurospheres and if the loss of TH expression in mesencephalic neurospheres is also accompanied by a loss in expression of other dopaminergic markers, semi-quantitative RT-PCR analysis were performed.

Explants from E12 cortices were initially mechanically dissociated and expanded in different growth factor combinations as illustrated in Figure 4-11A. ^{ctx}NS were expanded for 9 days and passaged once within this period before they were differentiated by growth factor withdrawal. After 6 days differentiation, the RNA was isolated and RT-PCR performed on DNAse treated samples. None of the growth factor combinations induced the expression of TH in ^{ctx}NS showing that ShhN and FGF8 alone are not sufficient to convert cortical precursor cells into TH expressing neurons. Nurr1, which is strongly linked to the differentiation of midbrain dopaminergic neurons (Saucedo-Cardenas et al., 1998), was expressed in the expanded ^{ctx}NS at low levels. However, no significant upregulation was observed as a result of potential synergistic effects of ShhN and FGF8. Very low levels of Ptx3 expression were detected. No differences in the expression levels of Ptx3 were evident for the various treatments. Overall, it was concluded that the morphogens ShhN and FGF8 did not induce an upregulation of dopamine neuron specific markers

in E12 cortical neurospheres and other midbrain specific factors may be required for their conversion into a dopaminergic phenotype.

^{mes}NS were expanded over four weeks with continuous passaging by mechanical dissociation of the neurospheres and proliferation under the various growth factor combinations as illustrated in Figure 4-11B. After two weeks expansion the neurospheres had been passaged three times and aliquots were used for RNA extraction and differentiation. As shown in previous experiments by immunocyochemistry, RT-PCR (Figure 4-11C) revealed an upregulation of TH expression in FGF8 expanded ^{mes}NS. This upregulation was detectable in samples analysed from proliferating cultures as well as from differentiated samples. ShhN present during the expansion phase further augmented the effects of FGF8 on TH expression. Cultures expanded in FGF2 showed a decreased TH expression compared to FGF8 expanded cultures even in the presence of ShhN confirming that the previously detected increased number of dopaminergic neurons is FGF8 dependent. Strikingly, a drop in TH expression was detected when ^{mes}NS were simultaneously expanded in ShhN, FGF8 and FGF2 as has been observed previously by immunolabelling. Ptx3 expression had a similar pattern as TH during the proliferation phase with the strongest expression in cultures that were expanded with FGF8 and ShhN. This confirmed that these conditions promoted the specific midbrain dopaminergic phenotype and had not merely upregulated TH expression, while additional FGF2 decreased the expression of this dopaminergic marker. The midbrain marker Engrailed-1 (En1), which acts in a pathway with wnt-1 signalling and FGF8 (Joyner, 1996; Danielian and McMahon, 1996), showed no differences in the expression levels between the different treatments.

The precursor cells were expanded and passaged for another three weeks. RT-PCR analysis was performed after five passages, a time point when no TH positive cells could be detected by immunochemical approaches. Indeed none of the cultures expressed detectable levels of TH, although Nurr1 expression appears to be upregulated in those conditions when ShhN and FGF8 were simultaneously present. Whether such an upregulation could reflect an inductive effect of the morphogens is speculative, particularly considering that these expression levels were not maintained

during the differentiation phase. This pattern was also not reproduced by Ptx3 expression showing decreased levels compared to samples prepared after 3 passages.

Overall, RT-PCR analysis revealed that after extended passaging of FGF8 expanded neurospheres, the mesencephalic precursors loose their ability to produce a dopaminergic phenotype. Morphogens such as FGF8 and ShhN that are beneficial for dopamine generation at early passages have no positive effects on the expression of dopaminergic markers at later passages. Under differentiation condition at later time points none of the midrain dopaminergic markers were expressed suggesting that under the chosen culture conditions no premature dopamine neuroblasts were generated that had only failed to differentiate into TH expressing dopaminergic neurons.

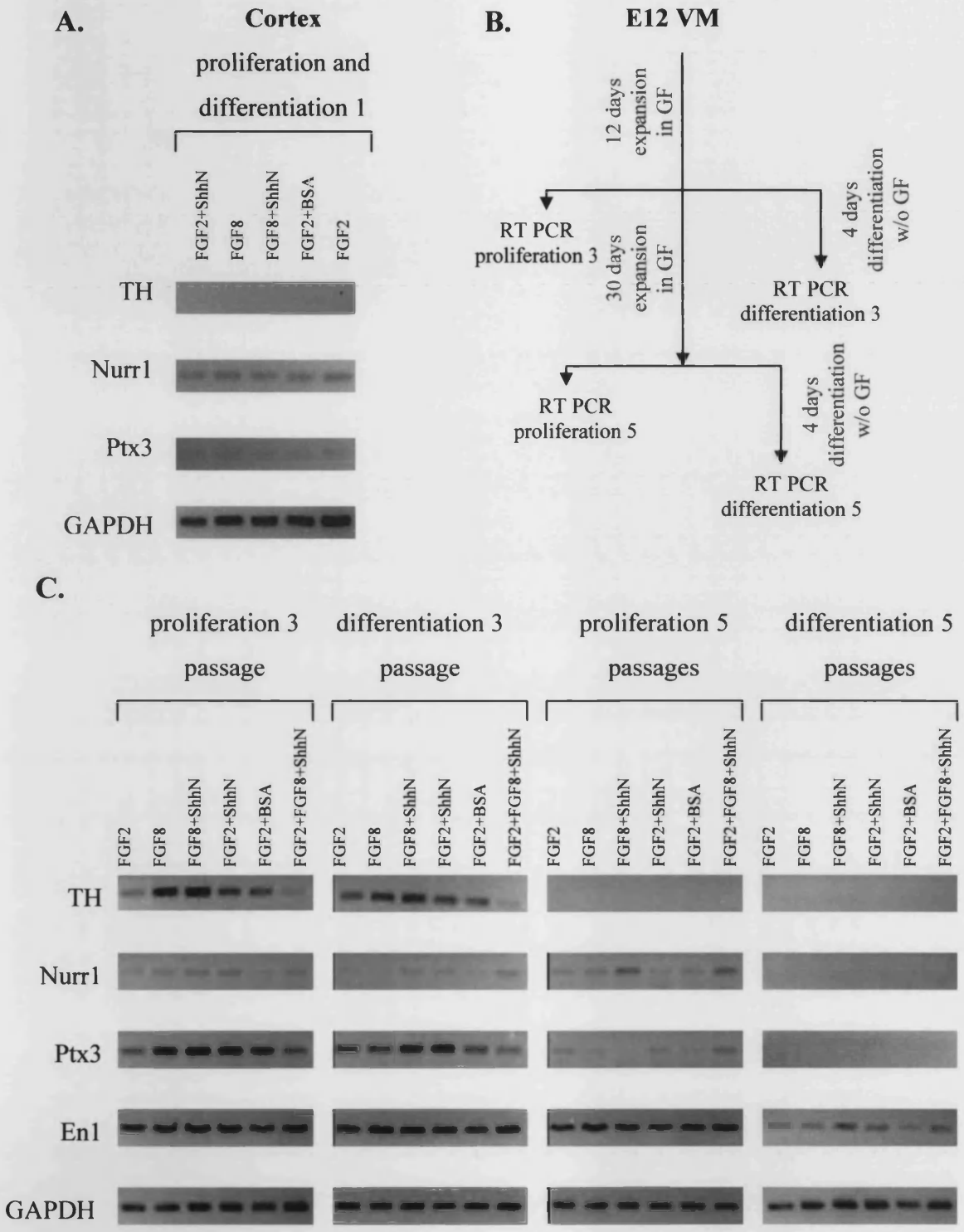
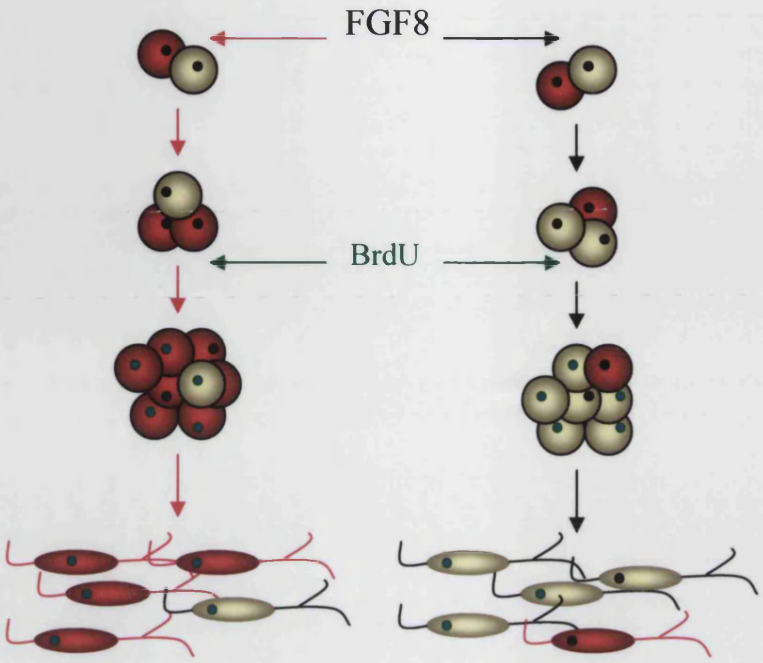


Figure 4-11: Semi-quantitative RT-PCR analysis of E12 neural precursors expanded in different growth factor combinations. (A) RT-PCR analysis of E12 ^{ctx}NS did not show an upregulation of dopaminergic markers. (B) Experimental rationale for RT-PCR of mesencephalic expanded precursors. GF= growth factor. (C) RT-PCR analysis of E12 ^{mes}NS for dopaminergic markers after long time expansion as described in the text.

4.3.8 Is FGF8 a survival factor for primary dopaminergic neurons or a mitogen for a dopamine neuroblast?

Although the previous data suggested that FGF8 is not inductive to convert a multipotent precursor cell into a dopamine progenitor, FGF8 might be mitogenic for an existing dopamine neuroblast and as such driving its cell division resulting in an increased number of *de novo* developed dopamine neurons. Alternatively, FGF8 might be a survival factor for existing primary dopaminergic neurons or fate restricted dopamine neuroblasts that undergo their terminal differentiation into mature dopaminergic neurons *in vitro*. To establish whether increased numbers of TH neurons arose from proliferating progenitors we designed a BrdU pulse experiment (Figure 4-12). FGF8 or FGF2 expanded ^{mes}NS were pulse labelled with BrdU and the number of BrdU/TH double labelled dopaminergic neurons determined after differentiation.

As shown in Figure 4-12, no statistically significant difference in the number of BrdU labelled dopaminergic neurons expanded in FGF8 or FGF2 was detected. At both time points the BrdU pulse resulted in a similar number of *de novo* developed dopaminergic neurons which lead to the suggestion that FGF8 is no more mitotic on dopamine precursor cells than FGF2. Thus, the increased number of dopaminergic neurons with FGF8 must have been generated from cell divisions before the cells were pulsed and most likely FGF8 increased their survival.



% BrdU/TH double labeled cells

	3-4 DIV	6-7 DIV
FGF2	7.2±2.0	7.0±1.8
FGF8	8.3±1.2	8.6±1.9

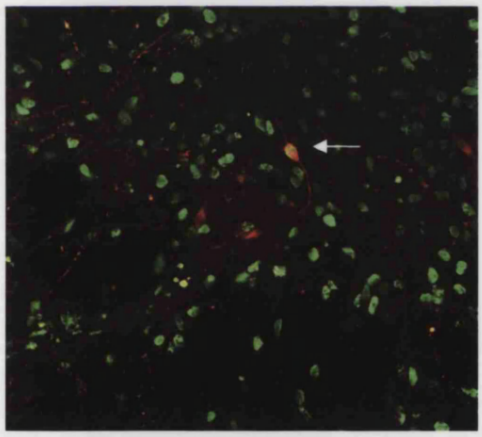


Figure 4-12: FGF8 is not a mitogen for a dopamine neuroblast but a survival factor. To answer the question if FGF8 is driving proliferation of a dopamine neuroblast or rather increases the survival of existing primary dopaminergic neurons or neuroblasts, a BrdU pulse experiment was performed. If FGF8 is a mitogen for dopamine neuroblast significantly more *de novo* developed dopaminergic neurons would be expected characterized as BrdU/TH+ve cells than with FGF2. However, the number of double labelled cells will be unchanged between the two mitogens if FGF8 increases the survival of post-mitotic dopamine neuroblasts. FGF8 or FGF2 expanded E12 ^{mes}NS were pulse labelled at the indicated time points with BrdU (0.2µM) for 12h and following differentiation stained for BrdU and TH. The number of BrdU/TH double labelled cells was not significantly increased when neurospheres were expanded in FGF8. Immunocyotchemical staining shows TH expressing cells (Alexa 546, red) that incorporated BrdU (Alexa 488, green) (arrow).

4.3.9 Effects of FGF8 on proliferation of endogenous stem cells of the SVZ

Proliferation of endogenous neural stem cells can be stimulated by delivery of exogenous growth factors, such as EGF and FGF2, into the lateral ventricle resulting in an increased division of stem cells residing in the SVZ of the forebrain (Craig et al., 1996; Kuhn et al., 1997). It was decided to use a similar approach to study the potential roles of FGF8 on proliferation of forebrain neural stem cells *in vivo* by delivering recombinant FGF8 with a replication-deficient HSV-1. Construction and expression of the vector RL1+/27+/4-pR19FGF8bwpre will be described in chapter 5.

The experimental rationale, as illustrated in Figure 4-13, comprised stereotaxic injections of FGF8 expressing virus (RL1+/27+/4-pR19FGF8bwpre) or control virus (RL1+/27+/4-pR19LacZ) into the lateral ventricle of adult Sprague-Dawley rats (n=4 per group). To label dividing cells, these injections were followed with daily intraperitoneal injections of BrdU at 100mg/kg for the next 18 days. On day 20, the animals were killed and the brains were fixed along with the olfactory bulbs. The brains were then sectioned and stained for BrdU in tandem with phenotype-specific markers (Figure 4-13).

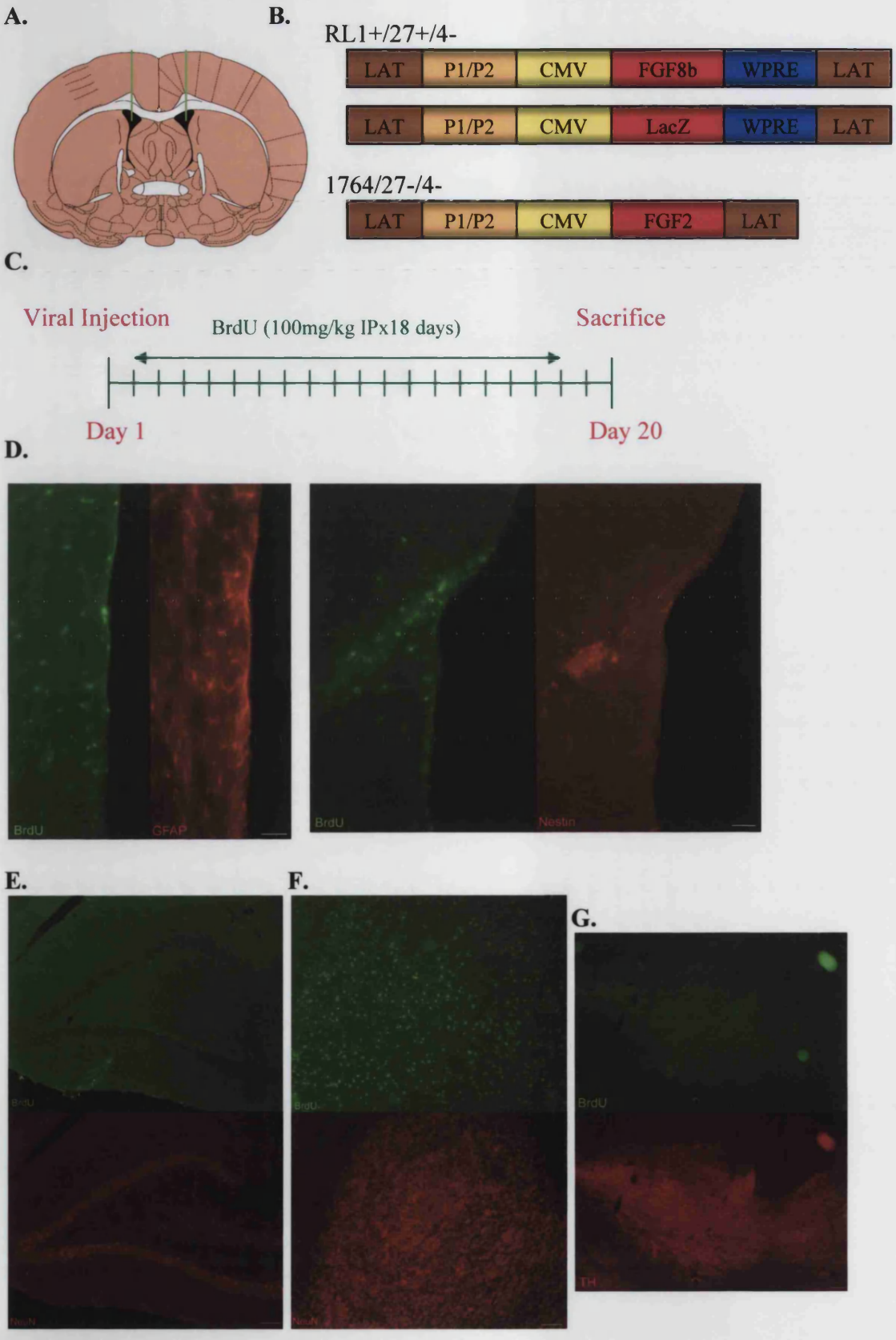
The effects RL1+/27+/4-pR19FGF8bwpre on proliferation of endogenous NSC's were first assessed within the SVZ around the injection site. No differences in the number of BrdU positive cells were evident in comparison with injections of the control virus. Within the subependymal layer only a low number of cells incorporated BrdU suggesting a slow and unaltered division of NSC's within this area. Some BrdU positive cells had migrated into the adjacent striatal areas. No upregulation of GFAP or nestin expression could be detected confirming that FGF8 had no effect on the cell population within the stem cell niche of the SVZ.

Next it was determined if FGF8 had any effects in other areas, such as olfactory bulb, hippocampus and substantia nigra. Although plenty of BrdU labelled cells were detected in the olfactory bulb, resulting from unaltered migration of dividing cells from the SVZ, no differences in the total neuronal number or the amount of cells

double labelled with the neuronal marker NeuN and BrdU were detected. Migration of BrdU incorporated cells was observed as a ridge leading from the caudal part of the lateral ventricle via the rostral migratory stream (RMS) to the olfactory bulb where cells radially dispersed (Figure 4-13H). Therefore, FGF8 did not appear to have any effects on survival, migration or differentiation of precursor cells from the SVZ to the olfactory bulb. Only a rare number of BrdU positive cells were found in the hippocampus and no evidence of cell division in the substantia nigra was apparent. A lack of dopamine neurogenesis in the substantia nigra is in agreement with a recent report (Frielingsdorf et al., 2004).

In order to confirm that the experimental rationale allowed studying the proliferation of endogenous stem cells after ventricular injections of growth factor expressing HSV-1, disabled HSV-1 expressing FGF2 was injected into the lateral ventricle. As shown in Figure 4-13 a massive proliferation within the subependymal layer of the SVZ was detected after injection of the FGF2 expressing virus compared to the control virus. In FGF2 injected animals "polyp-like" hyperplasia containing an increased number of BrdU positive cells were found. An accelerated number of BrdU positive cells were also observed in the olfactory bulb and striatum of FGF2 injected animals. This confirmed that the experimental rationale itself allowed induction of cell proliferation within the subependymal layer after viral delivery of recombinant FGF2. These observed proliferative effects after intraventricular delivery of FGF2 on endogenous precursor cells and their differentiation into neuronal cell types have been described elsewhere (Kuhn et al., 1997), and hence were not further investigated in this study.

Overall, it was concluded that under the chosen experimental rationale virally delivered FGF8 did not induce proliferation of endogenous neural stem cells generated from the SVZ and that it did not effect in any obvious way their migration or differentiation in the olfactory bulb.



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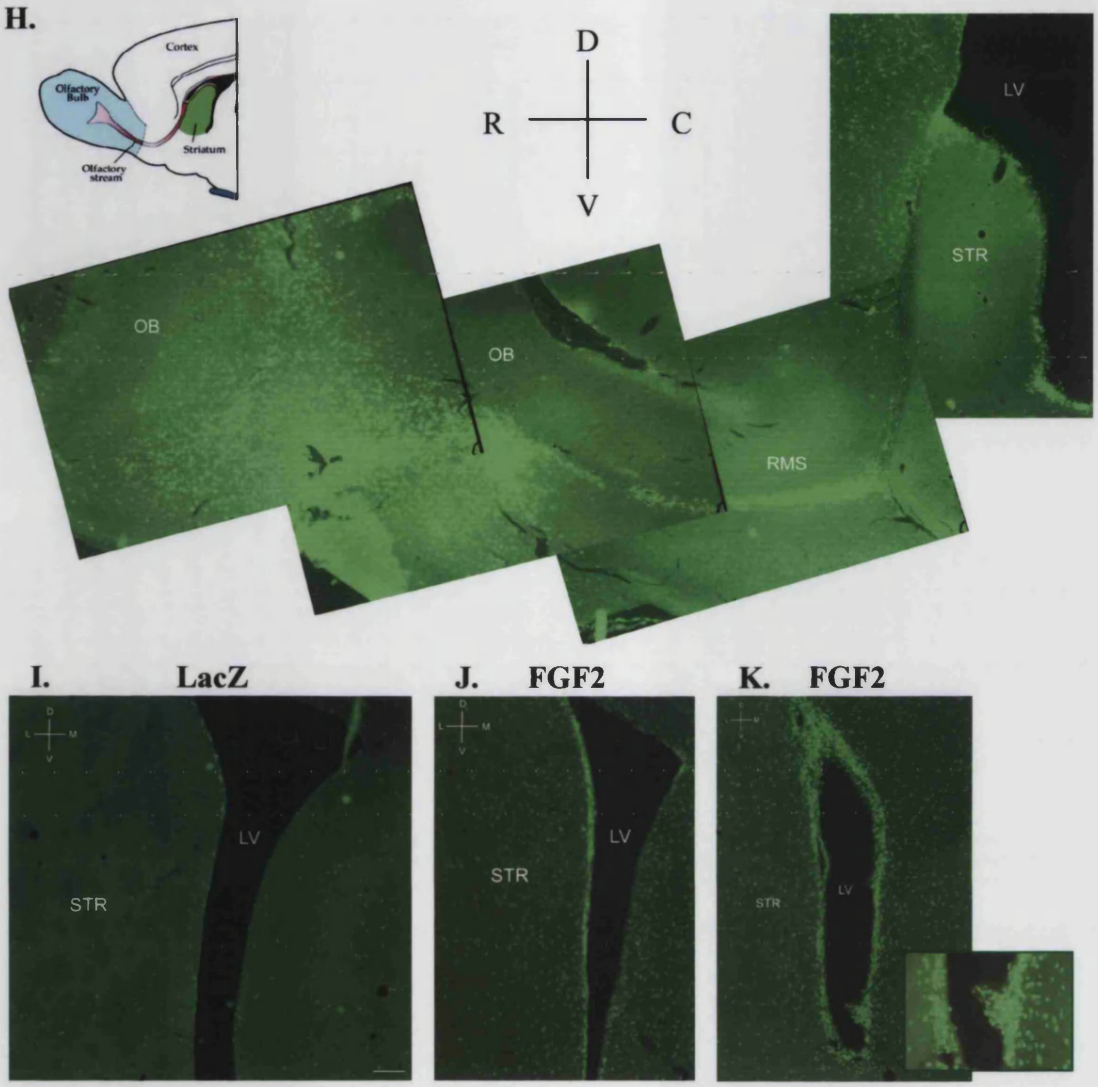


Figure 4-13 continuation: Virally delivered FGF8 has no effect on proliferation of endogenous precursor cells of the SVZ. Effects of virally delivered FGF8 were tested after (A) injections into both lateral ventricles of adult rats (n=4). (B) Viral constructs used were the less disabled backbone expressing either FGF8 or LacZ and the highly disabled construct expressing FGF2 as positive control. (C) Experimental rationale was to inject $5x10^6$ PFU per ventricle of viral vector followed by 18 days of BrdU injections (100mg/kg, i.p.) and sacrification of the animals two days after the last BrdU injection. (D) to (H) immunohistochemical stainings for the group injected with FGF8 expressing virus showed no significant increase in the number of BrdU positive cells. (D) No significant upregulation of GFAP or nestin expression was found in the SVZ of the lateral ventricle. Other regions analysed were (E) hippocampus, (F) olfactory bulb and (G) substantia nigra. No significant changes to the control group were observed. (H) BrdU labelled cells migrated from the SVZ of the lateral ventricle as rostral migratory stream (RMS) into the olfactory bulb (OB) where they disperse to differentiate into neurons. (I) Animals injected with LacZ expressing virus showed a similar number of BrdU positive cells around the lateral ventricle (LV). (J) and (K) animals injected with FGF2 expressing virus had a significant increased number of BrdU positive cells within the SVZ and the surrounding striatal tissue. (K) shows massive proliferation in the LV in caudal positions of the injection site and often hyperplasia (inset) were detected in FGF2 injected animals as "polyp-like" changes of the lateral wall containing an increased number of dividing cells. Scale bars represent 50µm in (D) and 100µm in (E) to (I).

4.4 Discussion

This chapter has shown that another member of the fibroblast growth factor family, FGF8b, is a potent mitogen for the expansion of neural stem cells derived from different regions of the developing CNS. The expanded NSC's retain definite stem cell characteristics as demonstrated by differentiation into the three neural lineages after subsequent passaging. Heparan sulphates generally play an important role for the activity of FGFs (Yamaguchi, 2001) and this has also been proven for the mitogenic activity of FGF8b (Loo and Salmivirta, 2002). Probably the lack of heparin in the culture conditions of other studies is the reason that the function of FGF8 on NSC's has not so far been seen. The presence of heparin was absolutely required for efficient and long time expansion of ^{mes}NS in this study, as has also been shown for mesencephalic precursor cells using FGF2 (Caldwell and Svendsen, 1998). Other studies that suggested the potential mitogenic effects of FGF8 on cortical (Hajihosseini and Dickson, 1999) and mesencephalic (Studer et al., 2000) precursors did not use heparin. However, these studies used cultures grown attached to a substrate rather than free-floating spheres which may be less dependent on exogenous heparin due to a continual production of heparin-like molecules. Further more, these studies did not grow the precursor cells as continuous cultures with subsequent passaging, and could therefore be considered as primary neural progenitor cultures.

Growth curves and dose response analysis revealed that FGF8 has a significantly lower mitogenic activity than FGF2. An appropriate ED_{50} of 60ng/ml for FGF8 compared to 10ng/ml for FGF2 was calculated based on their ability to stimulate BrdU incorporation in striatal neural precursor cells. Lower activities may reflect differences in their affinities for FGFR as it has been shown that FGF8 preferably binds to FGFR3 IIIc (Ornitz et al., 1996; Ford-Perriss et al., 2002), while FGF2 facilitates its mitogenic functions in neuroepithelial cells via FGFR1 (Brickman et al., 1995) but can also bind to FGFR3 in other cell lines (Ornitz et al., 1996). FGFR1 and FGFR3 as mediators for FGF2 and FGF8 signaling, respectively, are expressed in the developing brain between E10 and E14 (Ford-Perriss et al., 2002), the embryonic ages used in this study.

Activation of distinct receptors did not appear to have an impact on the outcome of progenitors generated as no differences in the composition of the neural progeny produced were observed. Of interest is the marginal shift within the glial progeny for mesencephalic neurospheres since the increase of oligendrocytes in FGF8 expanded cultures is accompanied by an identical decrease in the number of astrocytes compared to FGF2 expanded cultures (Figure 4-6). This leads to the speculation that FGF8 actually drives a common mesencephalic precursor cell into the oligodendroglial lineage at the cost of differentiation into astrocytes. Indeed it has been shown that FGFR3 signaling regulates terminal differentiation of oligodendrocyte progenitors (Oh et al., 2003b), and increased activation of this receptor may contribute to an augmented generation of oligodendrocytes in FGF8 expanded cultures.

Interestingly, mesencephalic neural precursor cells gave rise to significantly more dopaminergic neurons, characterized by the expression of TH, when expanded in FGF8 as compared to FGF2. The number of dopaminergic neurons could further be increased when ShhN was simultaneously present with FGF8 during the expansion phase of ^{mes}NS. That the upregulation of dopaminergic markers depends on the presence of ShhN during the expansion phase and that the morphogen may even depress TH expression when present during the differentiation phase has also been proven for dopaminergic neurons generated from adult hippocampal progenitors (Sakurada et al., 1999).

Three possible explanations for the augmented number of TH positive cells may be considered: a) FGF8 induces *de novo* development of dopaminergic neurons from multipotent precursor cells, which could collaboratively be increased by FGF8 and ShhN; b) FGF8 is a mitogen particularly for amplifying dopamine neuroblasts; c) FGF8 exerts survival effects on existing dopamine neuroblasts and/or differentiated primary dopaminergic neurons. Several observations disprove that FGF8 and ShhN were converting a multipotent neural stem cell into a dopaminergic progenitor cell as demonstrated during embryonic development (Ye et al., 1998) and for the generation of dopaminergic neurons from embryonic stem cells (Lee et al., 2000; Kim et al.,

2002). A neural stem cell that exists within the neurosphere and is susceptible to the morphogens should give rise to TH expressing cells when isolated at different embryonic ages and not loose its susceptibility to the signals following passaging. A reduced number of dopaminergic neurons in E14 ^{mes}NS compared to E12 ^{mes}NS were observed and a significant decline in the total number of TH positive cells with each passage. Further more, the morphogens did not induce upregulation of TH expression or other dopaminergic markers in cortical neural precursor cells or mesencephalic neurospheres that had been passaged over time. Other reports also confirmed that ShhN and FGF8 are not sufficient to induce a dopaminergic phenotype in mouse striatal precursor cells and in a human neural stem cell line (Stull and Iacovitti, 2001). It was also demonstrated that the effects of ShhN are not a unique phenomenon resulting from synergism with FGF8 but could also be mimicked with FGF2. However, the high concentration of FGF-2 used to propagate the NSC's (20ng/ml) is known to activate all three FGF receptors (Ornitz et al., 1996), suggesting that FGFR3 may be activated under the chosen conditions by FGF2. The BrdU pulse experiments performed suggest that FGF8 is not a specific mitogen for the proliferation of dopamine neuroblasts when comparing the *de novo* generated dopaminergic neurons to those in FGF2 conditions. Therefore, the increased number of TH expressing cells in FGF8 conditions did not result from the *de novo* generation of dopaminergic neurons but most likely due to survival effects on existing primary dopaminergic neurons and post-mitotic dopamine neuroblasts. Neuroprotective effects of FGF8 have also been reported for rat hippocampal neurons (Mark et al., 1999), PC12 cells and human neural progenitor cells (Tanaka et al., 2001).

Interestingly, a depression of TH expression was detected by immunocytochemistry and RT-PCR analysis when ^{mes}NS were simultaneously expanded in ShhN, FGF8 and FGF2. Considering that these factors are mitogens, they determine cell fates in a concentration dependent pattern. Therefore, excessive activation of FGFR3 or other FGFRs via FGF8 and FGF2 could actually inhibit dopamine differentiation and favour differentiation into other neuronal cell types. It has been shown that FGF8 and ShhN also induce serotonergic neurons in collaboration with FGF4 and exposure of midbrain explants to FGF2 or FGF4 gave rise to serotonergic neurons at the expense of dopaminergic neurons (Ye et al., 1998), while blocking of FGF4 signalling

increased the production of dopaminergic neurons on the cost of serotonergic neurons (Rodriguez-Pallares et al., 2003). Considering that different FGFs can transduce through the same cellular receptors (Ornitz et al., 1996) it is possible that at the high concentration of the mitogens used, FGF4-mediated signalling pathways are activated that may inhibit differentiation into mature dopaminergic neurons. Further, it has recently been shown that the trophic effects of FGF8 are dose dependent and that accelerated FGF8 signalling actually inhibits the FGF8-dependent cell-survival pathway (Storm et al., 2003). It was decided to use FGF8 at a concentration of 50ng/ml corresponding to the calculated ED_{50} value. Whether different FGF8 concentrations have effects on the number of dopaminergic neurons generated needs to be addressed in future studies.

Although FGF8 has mitogenic effects on precursor cells *in vitro*, no effects on proliferation of neural stem cells *in vivo* were observed after intraventricular delivery using disabled HSV-1. Several possibilities may explain this finding. Gene delivery with the chosen vector may be unsatisfactory either due to limited transduction efficiency of cells within the SVZ or low expression levels from the construct *in vivo* that are not sufficient for a biological effect. The previous chapter had shown that the selected viral backbone does infect cells of the SVZ. However, transgene expression was only observed in a limited number of subependymal cells. As shown by intraventricular injections of the FGF2 expressing vector, viral delivery is sufficient to induce a proliferative effect with FGF2. It should be considered that significantly higher concentrations of FGF8 may be required to induce a potential effect with FGF8 *in vivo*, as *in vitro* analysis had already revealed that the ED_{50} of FGF8 is significantly higher than that of FGF2. However, the simplest explanation is that no FGF8 responsive neural stem cell exists in the adult forebrain and that the mitogenic effects are limited to neural stem cells derived from the developing CNS. Expression of FGF8 (Tanaka et al., 2001) and its receptor FGFR3 (Belluardo et al., 1997) were confirmed in the adult rodent brain and FGFR3 expression was also localized in astrocytes within the postnatal ventricular zone (Pringle et al., 2003), but the function of FGF8 is likely to be very different from its mitogenic activity in the developing brain. Similarly, as has been shown for ShhN (Lai et al., 2002), FGF8 may effect proliferation or survival of hippocampal neural stem cells or be involved in

"maintaining" the stem cell niche as was reported for smoothened (Machold et al., 2003). In this study it was decided not to test if neural precursor cells derived from the adult SVZ or SGZ can be propagated as neurosphere cultures *in vitro*. However, it was found that neural stem cells isolated from human embryonic tissue (cortex, striatum, mesencephalon and spinal cord) could also be expanded in the presence of FGF8 as the sole mitogen (data not shown). It was also found that the presence of exogenous heparin is required to enhance the mitogenic effects of FGF8 and thus, insufficient amounts of heparin or heparin sulphates within the stem cell niche may further limit the function of FGF8 *in vivo*. As the identity and cellular properties of endogenous neural stem cells remain unclear, future studies will hopefully unveil information about the expression of the receptors and proteoglycans within this niche.

CHAPTER 5:

CONSTRUCTION AND CHARACTERIZATION OF HSV-1 VECTORS FOR THE POTENTIAL INDUCTION OF A DOPAMINERGIC PHENOTYPE

5.1 Introduction

This chapter describes the construction of disabled HSV-1 for the delivery of modulatory genes to neural progenitor cells, neurospheres and for potential *in vivo* applications. The aim was to study the effects of expression of selected factors on the induction of dopaminergic phenotypes in these models.

As described in the general introduction embryonic dopamine neuron development involves synergistic function of mitogenic, morphogenic and transcription factors. This chapter focuses on the roles of the mitogen FGF2, the morphogens FGF8 and ShhN and the transcription factor Nurr1. These selected factors have been well established as playing crucial roles in the development of dopaminergic neurons during embryonic development (Perrone-Capano and Di Porzio, 2000; Perrone-Capano et al., 2000) and for the induction of a dopaminergic phenotype in embryonic stem cells (for review see Lin and Rosenthal, 2003). Since functional FGF2, FGF8 and ShhN can be added to *in vitro* cell cultures as bacterially produced recombinant proteins, most studies have avoided the use of proteins produced in mammalian cell culture systems, or the construction of viral vectors expressing these factors. However, such bacterially produced recombinant factors have the disadvantage of the lack of posttranslational modification. The factors under study do undergo such modifications, e.g. FGF8 is glycosylated (Crossley and Martin, 1995) in mammalian cells and Shh is modified by cholesterol and palmitolyl attachment as well as forming disulfide bonds (see below). Further it has been shown that FGF2 and ShhN remain cell associated and that signalling pathways involve direct cell to cell contact (Ornitz and Itoh, 2001; Marti and Bovolenta, 2002). Hence, the mitogenic or differentiation activity of bacterial produced factors may only reflect a limited function of these factors. Given the important role of the interaction with proteoglycans, as shown in the previous chapters, these modifications may have significant relevance for their biological function *in vitro* and *in vivo*. Therefore, replication deficient HSV-1 vectors were constructed to deliver these factors to cell and animal models which should overcome all the limitations of the artificial production of cell free protein.

As established in chapter 3, the highly disabled 1764/27-/4- vector only gave low level gene expression in primary progenitor cells after infection at early time points. The less disabled backbone RL1+/27+/4- resulted in higher level gene delivery, although at the expense of increased neuronal cell loss. As it was intended to modify the function of progenitor cells at time points at which they still possess plasticity, i.e. when the cell population still contains immature precursor cells, gene delivery is required at early time points. Thus, the vector chosen was based on the RL1+/27+/4- backbone. The second model used in the study used neurospheres as the source for neural precursor cells. While striatal and mesencephalic derived neurospheres can be efficiently transduced with either viral vector, gene delivery to cortical neurospheres can only be achieved with RL1+/27+/4- viruses. However, infection with this construct at high multiplicities affected the differentiation capability of transduced precursor cells into neuronal cell types. Hence, for the delivery of genes encoding the selected factors to striatal and mesencephalic neurospheres the more highly disabled vector, 1764/27-/4-, may be more appropriate. Previous work has also shown that 1764/27-/4- is particularly suitable for *in vivo* gene delivery to the substantia nigra (Lilley et al., 2001). Intrastriatal administration results in a retrograde transport of the virus to the substantia nigra giving strong transgene expression in this region and around the striatal injection site. Therefore, 1764/27-/4- based vectors will be used in this chapter for *in vivo* gene delivery studies.

The genes encoding the respective factors were cloned into the pR19 expression cassette and flanked by sequences of the LAT region. This allows the targeted insertion of the expression cassette into the LAT region by homologous recombination, thereby replacing the endogenous LAT region. The rationale for the use of this cassette was based on previous work in where the pR19 based viruses had been shown to direct high level, long term expression of reporter genes *in vitro* and *in vivo* (Palmer et al., 2000; Lilley et al., 2001).

In an attempt to study the pathogenesis and possible therapeutic strategies for Parkinson's disease, several cell and animal models have been developed (Deumens et al., 2002; Dauer and Przedborski, 2003; Jakel et al., 2004). Animal models using rodents, primates and even drosophila (Feany and Bender, 2000) can be distinguished

as "etiological models" using gene-based approaches and "pathological models" that induce a selective degeneration or depletion of dopaminergic neurons by administration of toxins (Dauer and Przedborski, 2003). Most commonly used animal models were based on acute or chronic exposure to such toxins as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinine (MPTP), 6-hydroxydopamine (6-OHDA), paraquat and rotenone that induce oxidative stress by formation of reactive oxygen species (ROS) and/or inhibition of mitochondrial function (Jakel et al., 2004). Although these models imitate some characteristics of the disease they do not reflect the progressive degeneration which is symptomatic for the human pathogenesis.

For the purpose of this study a toxin-based animal model of PD was used that is easy to establish in rats, induces a profound and selective depletion of dopaminergic neurons within a reasonable time, and allows quantification. Therefore, it was decided to induce acute dopaminergic cell death by administration of 6-OHDA. 6-OHDA is selectively taken up by catecholaminergic neurons (Luthman et al., 1989) and induces neuronal cell death within the first 24 hours when injected into the medial forebrain bundle (MFB), which carries ascending dopaminergic and serotonergic projections to the forebrain, or into the substantia nigra (Jeon et al., 1995). However, when injected into the striatum it induces retrograde loss of dopaminergic neurons which is slower and progressive and lasts for 1 to 3 weeks (Przedborski et al., 1995). In this study the MFB was lesioned by unilateral injections of 6-OHDA which resulted in an almost complete loss of dopaminergic neurons in the ipsilateral substantia nigra and TH reactive fibers in the striatum. The advantage of unilateral lesions is that they can easily be measured by drug-induced asymmetric circling behaviour, the magnitude of which depends on the degree of the nigrostriatal lesion (Ungerstedt, 1971b). The postsynaptic agonist apomorphine induces rotations contralateral to the lesioned side due to a stimulation of denervation-induced upregulated D2 receptors in the ipsilateral striatum (Ungerstedt, 1971a). Apomorphine induced rotations become detectable when more than 90% of dopaminergic fibers in the striatum and more than 50% of dopaminergic cell bodies in the substantia nigra have been depleted (Deumens et al., 2002). This Parkinson model will be used to study the effects of selected virally delivered factors on the potential regeneration of the denervated nigrostriatal system.

5.2 Materials and Methods

5.2.1 Construction of FGF8b expressing viral vectors

The cDNA encoding for mouse FGF8b (GeneBank accession number D12482) was obtained in pBluescript® SK and the construction of the plasmid has been described before (Crossley and Martin, 1995). FGF8b cDNA was removed by restriction digest with SmaI and XhoI and then subcloned into the pR19 shuttle plasmid flanked by LAT sequences allowing insertion into the LAT region. Therefore, the reporter genes of the vector backbones pGEM-5 pR19LacZ LAT (Lilley et al., 2001) or pGEM-5 pR19Nurr1wpre LAT (constructed by J. Palmer, unpublished data), were removed by enzymatic digest with HindIII followed by blunt ending with T4 DNA polymerase (2.2.8) and restriction digest with XhoI. After ligation FGF8b containing clones were characterized by multiple restriction digests.

For the generation of recombinant viruses (2.4.1) 1764/27-/4-pR19GFP or RL1+/27+/4-pR19GFPwpre viral DNA, respectively, were co-transfected with linearised pGEM-5 pR19FGF8b LAT and pGEM-5 pR19FGF8bwpre LAT plasmid DNA. Recombinant plaques were detected by the lack of GFP expression under a fluorescent microscope and selected until pure stock of virus was obtained generating the viruses 1764/27-/4-pR19FGF8b and RL1+/27+/4-pR19FGF8bwpre, respectively.

5.2.2 Construction of FGF2 (bFGF) expressing viral vectors

The cDNA encoding for rat basic fibroblast growth factor was amplified from rat brain mRNA (2.2.12) by RT-PCR using the One-step RT-PCR kit (Qiagen). 2μg RNA were used as template in the RT-PCR reaction with the following primers nesting a HindIII restriction site in the 5' and a XhoI restriction site in the 3' end encompassing the ORF from 533bp to 1024bp according to the GeneBank accession number M22427:

FW: 5' CGTA*AAGCTT*GATTCCATGGCTGCCGGCAGCATCA

RV: 5' CGTA*CTCGAG*ATTCCCTGAGAGTGACAGTGTCTA

Restriction sites are indicated in Italic style.

The following RT-PCR program was used on a thermocycler (Eppendorf): reverse transcription for 30min at 50°C, PCR activation for 15min at 95°C, 34 cycles of denaturation for 1min at 95°C, annealing for 1min at 80°C, extension for 1min at 72°C followed by a final extension for 10min at 72°C. The PCR product was separated on a 1% agarose gel, the band of the expected molecular size (about 520bp) cut out and purified using a Gel band purification kit (Amersham). The PCR product was subloned into pgemT vector (Promega) resulting in pGemTbFGF. Insert containing clones were sequenced (2.2.11) and the sequence compared to the Genebank sequence of bFGF (M22427). The sequence is attached in the appendix.

The cDNA encoding for bFGF was removed from pGemTbFGF by restriction digest with HindIII and XhoI and subloned into a shuttle vector to replace the transgene of pGEM-5pR19LacZLAT or pGEM-5pR19Nurr1wpreLAT within the HindIII and XhoI sites. The plasmids generated were named pGEM-5pR19bFGFLAT and pGEM-5pR19bFGFwpreLAT and subsequently linearised with SspI.

For generation of bFGF expressing viruses, the linearised plasmids were co-transfected with viral DNA generated from 1764/27-/4-pR19GFP and RL1+/27+/4-pR19GFPwpre, respectively. Recombinant plaques were detected by the lack of GFP expression under the fluorescence microscope, selected until pure and the constructed viruses named 1764/27-/4-pR19bFGF and RL1+/27+/4-pR19bFGFwpre.

5.2.3 Construction of ShhN expressing viral vectors

ShhN was amplified by PCR from rat embryonic cDNA (Quick clone, clontech) using 0.1ng of the template and the following program: hot start for 2min at 95°C, 34 cycles of denaturation for 1.3min at 95°C, annealing for 1.45min at 69.8°C, extension for 2min at 72°C and final extension for 10min at 72°C. The primers were designed to amplify the aminoterminal part of rat sonic hedgehog (amino acid residues 1-198 according to Gene Bank accession number L27340). Therefore, a stop codon was

inserted in the reverse primer after 908bp (according to #L27340) and is indicated (underlined) in the following sequence:

FW: 5' CGTA*GGATCCGAATTC*CGTACCAGCTCGCGCACAGA

RV: 5' CGT*ACTCGAGAATTC*TCAGCCGTCAGATTTGGCCGCCA

Further the primers contained restriction sites for BamHI and EcoRI (FW) or EcoRI and XhoI (RW) as indicated in italic style. The PCR reaction resulted in a 753bp large product that was subsequently cloned into pgemT vector (Promega) resulting in pgemTShhN. The sequence of correct clones was confirmed and is attached in the appendix.

The cDNA encoding for ShhN was removed by restriction digest with BamHI followed by blunt ending with T4 DNA polymerase (2.2.8) and XhoI digest. The fragment was cloned into a shuttle vector to replace the transgene of pGEM-5pR19LacZLAT or pGEM-5pR19Nurr1wpreLAT. Therefore, the plasmid was HindIII digested, blunt ended with T4 DNA polymerase and the insert subsequently removed by XhoI digest. The plasmids generated were named pGEM-5pR19ShhNLAT and pGEM-5pR19ShhNwpreLAT and subsequently linearised with SspI.

Recombinant viruses were generated by co-transfection of linearised plasmids with viral DNA from 1764/27-/4-pR19GFP (pGEM-5pR19ShhNwpreLAT) and RL1+/27+/4-pR19GFPwpre (pGEM-5pR19ShhNwpreLAT). Recombinant plaques were detected by the lack of GFP expression under the fluorescence microscope, selected until pure and the constructed viruses named 1764/27-/4-pR19ShhN and RL1+/27+/4-pR19ShhNwpre.

5.2.4 Construction of Nurr1 expressing viral vectors

Nurr1 was amplified by PCR from rat embryonic cDNA (Quick clone, clontech) using 0.1ng of the template and the following program: hot start for 2min at 95°C, 34 cycles of denaturation for 1.3min at 95°C, annealing for 1.45min at 69.8°C, extension for 2min at 72°C and final extension for 10min at 72°C. Primers were designed inserting an additional HindIII restriction site in the 5'end and an XhoI site in the 3'end with the following sequence (restriction sites in italic style):

FW: 5' CGTA*AAGCT*TTCGGCTGAAGCCATGCCTTG

RV: 5' CGTA*CTCGAG*ACGTGCATGGGAGAAAGTC

The resulting PCR product of about 1.8kbp was cloned into the pgemTeasy vector (Promega) resulting in pGemTNurr1. The sequence of selected clones was determined and verified with the published entry (Gene Bank accession number U72345). Nurr1 was subcloned into the expression cassette by using the Hind III and XhoI restriction sites available in the vector pGEM-5pR19LacZLAT and thereby replacing the LacZ gene. The resulting vector is referred to as pGEM-5pR19Nurr1LAT. For generation of viruses expressing the transgene with the wood chuck hepatitis virus posttranscriptional regulatory element, the Nurr1 gene was subcloned by others of our group into pGEM-5pR19hrGFPwpreLAT (Palmer J, personal reference). Therefore, the hrGFP gene was replaced by Nurr1 using HindIII and NotI restriction sites generating pGEM-5pR19Nurr1wpreLAT (Palmer J, personal reference).

Recombinant virus were generated by co-transfection of 1764/27-/4-pR19GFP and pGEM-5pR19Nurr1LAT, resulting in 1764/27-/4-pR19Nurr1, or RL1+/27+/4-pR19GFPwpre and pGEM-5pR19Nurr1wpreLAT, resulting in RL1+/27+/4-pR19Nurr1wpre. Recombinant plaques were identified under a fluorescent microscope by the lack of GFP expression and subsequently selected until pure.

5.2.5 Western blot analysis

BHK cells, primary E14 cortical, striatal and mesencephalic neural progenitors and neurosphere cultures were infected with the indicated viral constructs at an approximate m.o.i. of 10. For secreted proteins infected BHK cells were maintained for two days in standard growth medium, then the media was removed and the cells washed three times with HBSS (Gibco). Cells were overlaid with 500μl DMEM medium (SFM) and incubated at 37°C/5%CO_2. After 2, 6 and 24h the supernatant was collected and centrifuged for 2min at 3000rpm. The supernatant was concentrated using Microcon columns (Millipore, cut off 10 kDa) and resuspended in a small volume (25-30μl) of western blotting loading buffer containing 0.1M DTT. BHK infected cell pellets as well as infected progenitor cells were harvested in 100μl

loading buffer. Infected neurosphere cultures were maintained for five days after infection in differentiation media. Cells were settled and resuspended in 100μl loading buffer. All western blotting samples were stored at -20°C. SDS-PAGE, transfer and antibody incubations were performed as described in 2.5.1.

5.2.6 Neurite outgrowth assay

6000 viable PC12 cells (2.3.4) were seeded per well into six-well multiwell plate (Nunclon) coated with poly-L-lysine. The next day cells were infected by removing growth media and incubation with 500μl DMEM containing 1.8x10^6PFU of the respective viral construct for one hour at 37°C. The virus was removed and cells overlaid with maintenance medium. Positive control cultures (only for neurite assay of 1764/27-/4- constructs) were supplemented with recombinant FGF8 (R&D, 50ng/ml) or FGF2 (R&D, 5ng/ml) and growth factors were freshly added every other days at the indicated final concentration. Three days after infection medium was removed and replaced by DMEM containing reduced serum (2%FCS only). Six days after infection the cells were fixed with 4% paraformaldehyde. For quantification cells were counted in 20 fields per treatment with processes of the length of the cell diameter or longer. The neurite outgrowth assay was repeated in three independent experiments.

5.2.7 Differentiation of C3H/10T1/2 cells

For the standard assay of ShhN activity 1x10^4 viable mouse fibroblast C3H/10T1/2 cells/cm^2 were plated in 8-well chamber slides (Nunclon). The next day medium was removed and cells infected with 100μl of virus diluted in SFM. Cells were infected at an m.o.i. of 3 with 1764/27-/4-pR19ShhN and RL1+/27+/4-pR19ShhNwpre or the respective viral backbones expressing GFP. After one hour infection at 37°C /5%CO_2 virus was removed and replaced by 250μl DMEM with reduced serum content (3%FCS). Cells were maintained for another five days and subsequently fixed with 4% paraformaldehyde. Cells were washed three times with Hank's balanced solution (HBSS, Gibco). Infected cells were assayed for alkaline phosphatase (AP) by adding 200μl 5-Bromo-4-chloro-3-indolyl Phosphate (BCIP)/Nitroblue Tetrazolium (NBT)

Liquid Substrate (BCIP/NBT) (B1911, Sigma) per well. Cells were incubated at room temperature and colour development monitored under a standard microscope. AP activity produced a blue-purple, stable precipitate in the cells. Colour development was stopped by rinsing three times with distilled water.

5.2.8 6-OHDA lesions

6-hydroxydopamine Hydrochloride (6-OHDA, Sigma 14381) was freshly prepared in 0.9% saline solution containing 0.2% ascorbic acid at a concentration of 2.5μg/μl. Aliquots were protected from exposure to light and kept on ice prior to injection. Adult female Sprague-Dawley rats (260-280g) were anaesthetised and fixed in a Kopf stereotaxic frame. Under standard surgical procedure (2.6.1) 12.5μg (total volume of 5μl) of free base 6-OHDA were injected at a rate of 1μl/min with a 32"gauge steel needle (Hamilton, #79628) attached to a 25μl Hamilton syringe using an automatic microinjector (World Precision Instruments). Injections were performed into the left medial forebrain bundle at the following coordinates (Paxinos and Watson, 1997): AP-0.22, ML+0.15 from Bregma and DV -0.79 from dura with the incisor bar adjusted that bregma and lambda were positioned in a horizontal line (flat-skull position). After injection the needle was left in place for another 5min before slowly retracting it within 3min.

5.2.9 Behavioural analysis

At two weeks after lesion the drug-induced rotational behaviour was monitored for the first time in automated rotometer bowls (MedAssociates, USA). Apomorphine HCL (Sigma) was freshly prepared in distilled autoclaved water at a concentration of 0.5mg/ml and protected from exposure to light. Animals were injected with apomorphine at 0.5mg/kg s.c., transfered into the rotational bowl and connected to the automatic counter (Rotorat™, MedAssociates, USA). 5min after apomorphine injection net rotational asymmetry was monitored over 30 min as full contralateral body turns/min. Animals showing 5 or more turns were selected and apomorphine induced rotation tests were repeated after 4 weeks and 6 weeks prior to virus injection and after virus delivery as indicated.

5.2.10 Striatal virus injections

The GDNF expressing construct 1764/27-/4-pR19GDNF used in this study was previously constructed in our laboratory (Li XP, personal reference) and expresses the gene encoding for human GDNF in the pR19 cassette inserted in the LAT region of 1764/27-/4- backbone.

4 weeks (GDNF experiment) to 6 weeks (ShhN/FGF2 experiment) after lesion, apopmorphine induced rotational behaviour was assessed and one day later virus injections performed. Using standard surgical procedure (2.6.1) $5x10^5$ PFU of 1764/27-/4-pR19LacZ, 1764/27-/4-pR19FGF2, 1764/27-/4-pR19ShhN or 1764/27-/4-pR19GDNF were injected in a total volume of 5μl into the left striatum ipsilateral to the 6-OHDA lesion at the following coordinates (Paxinos and Watson, 1997): AP+0.18, ML+0.3 from Bregma and DV-0.45mm from dura with the incisor bar at +4.5mm from interaural line. For injection a 33" gauge steel needle (Hamilton, #79628) was attached to a 25μl Hamilton syringe and virus continuously injected at a rate of 0.5μl/min using an automated microinjector.

For BrdU labelling animals were injected i.p. with 100mg/kg BrdU in 0.1MTris/HCl pH7.4 daily for six consecutive days. Seven days after the last BrdU injection the animals were transcardially perfused with 4%PFA in 0.1M phosphatebuffer pH7.4, postfixed overnight and cryprotected in 30% sucrose until brains had sunk. Brains were cut in coronal sections at 30μm and immunohistochemical staining performed for BrdU, TH and nestin as described in 2.5.3.

5.3 Results

5.3.1 Overview of the vectors constructed

HSV-1 vectors were constructed expressing FGF8b, FGF2, ShhN and Nurr1 in either the highly disabled backbone 1764/27-/4- or the less disabled vector RL1+/27+/4- (Table 5-1 and Figure 5-1). While FGF8 expressing vectors were generated based on an existing plasmid encoding the mouse specific sequence of the gene (Crossley and Martin, 1995), all other genes were amplified using PCR methods from embryonic rat cDNA (Nurr1, ShhN) or adult tissue (FGF2) that was prepared for this purpose from rat brains. The correct sequences of all PCR based constructs were confirmed and are attached in the appendix.

Virus name	Deletions/ Inactivations	Transgene	Gene Bank Accession number
1764/27-/4-/pR19FGF2	ICP34.5/ORF P, VP16, ICP27, ICP4	rat basic fibroblast growth factor	M22427
1764/27-/4-/pR19FGF8	ICP34.5/ORF P, VP16, ICP27, ICP4	mouse fibroblast growth factor 8b	D12482
1764/27-/4-/pR19ShhN	ICP34.5/ORF P, VP16, ICP27, ICP4	rat sonic hedgehog (residues 1-198)	L27340
1764/27-/4-/pR19Nurr1	ICP34.5/ORF P, VP16, ICP27, ICP4	rat orphan nuclear receptor Nurr1	U72345
RL1+/27+/4-pR19FGF2	VP16, ICP4	rat basic fibroblast growth factor	M22427
RL1+/27+/4-pR19FGF8	VP16, ICP4	mouse fibroblast growth factor 8b	D12482
RL1+/27+/4-pR19ShhN	VP16, ICP4	rat sonic hedgehog (residues 1-198)	L27340
RL1+/27+/4-pR19Nurr1	VP16, ICP4	rat orphan nuclear receptor Nurr1	U72345

Table 5-1: Recombinant viruses constructed. All transgenes were cloned into the pR19 expression cassette and inserted into the LAT region.

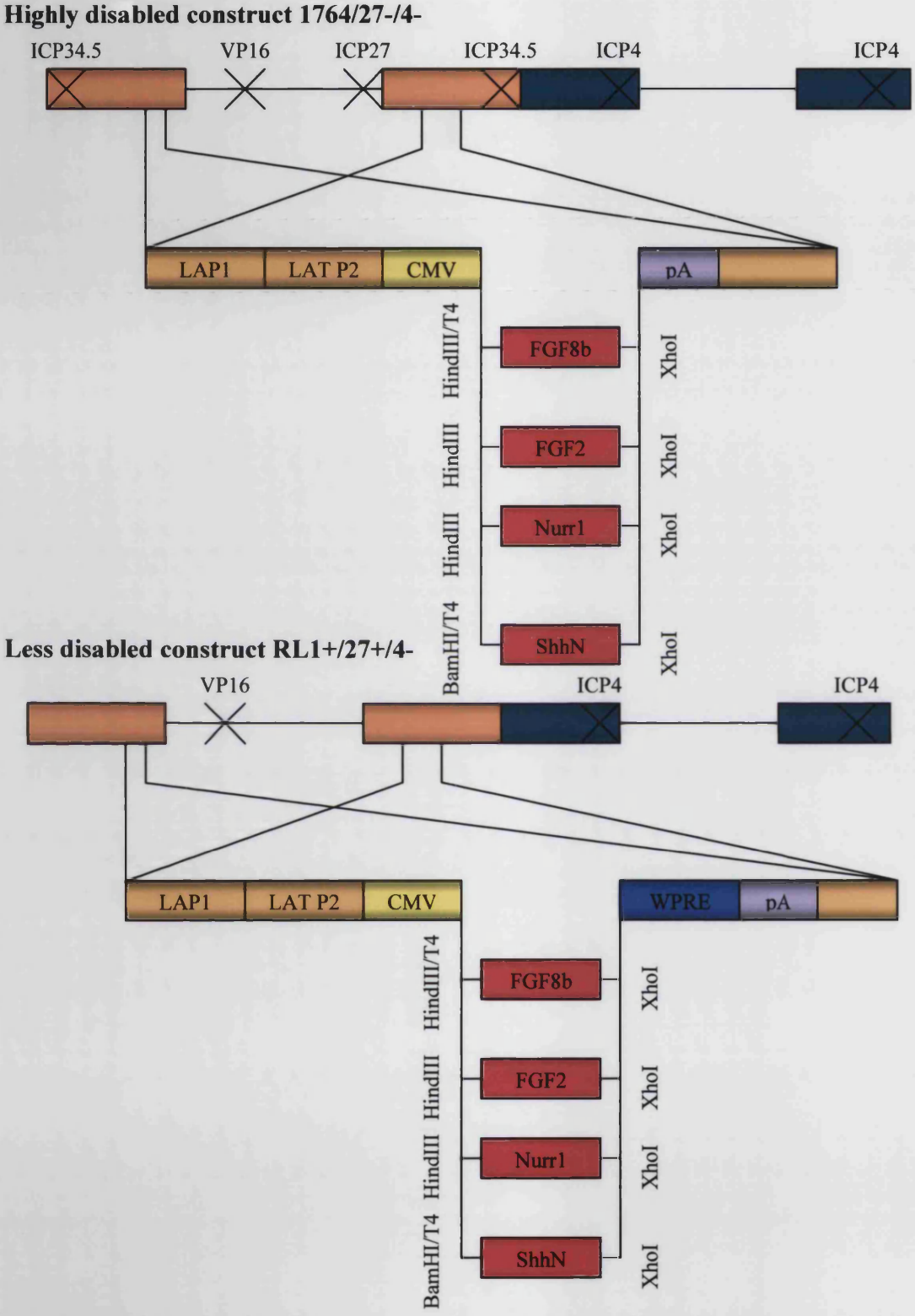


Figure 5-1: Genome structure of the viruses constructed. The cDNA encoding the respective factors were cloned into the pR19 expression cassette consisting of a heterologous promoter encompassing the HSV-1 latency promoter LAP1 and LAT P2 region combined with CMV promoter driving the transgene. The expression cassette was inserted into the LAT region of the respective viral backbones replacing the endogenous LAT elements.

5.3.2 The effects of virally delivered FGF8 and FGF2 in primary neuronal cultures and neurospheres

The effects of FGF8 and FGF2 on the generation and survival of dopaminergic neurons from neural precursor cells grown as neurospheres have been shown in the previous chapter. Here the effects of virally delivered FGF2 and FGF8 on primary neurons and neural progenitor cells were studied.

5.3.2.1 Construction of HSV-1 vectors expressing FGF8 and FGF2

Co-transfection of 1764/27-/4-pR19GFPLAT or RL1+/27+/4-pR19GFPwpreLAT viral DNA with linearised plasmid DNA containing FGF8b or FGF2 either with or without the woodchuck hepatitis virus post-transcriptional regulation element (wpre) resulted in replacement of the GFP expressing promoter cassette by FGF8 and FGF2 encoding cassettes, respectively. Homologous recombination can either occur in one or both sites of the LAT region generating faint or no GFP expressing viral plaques. Therefore, generation of recombinant plaques replaced for both LAT regions are stochastically rarer than those with unilateral recombination. Indeed, only a small number of recombinant plaques with both LAT regions replaced were detected as characterized by the absence of GFP expression. Faint GFP expressing viral plaques were observed but not selected for further virus purification. Plaques based on the less disabled backbone, RL1+/27+/4-, were significantly larger than those generated from highly disabled vector, 1764/27-/4-, since the latter constructs have decreased growth rates due to the multiple viral gene deletions. Purified recombinant constructs that did not contain any GFP expressing particles were tested for their lack of growth on non-complementing BHK cells.

To select positive recombinant vectors, purified clones were first tested for their expression of FGF8 or FGF2 on 27/12/M:4 (M49) cells (Thomas et al., 1999b), which allow virus growth. Most of the purified clones expressed FGF8b (Figure 5-2A), while some of the isolates did not express FGF2 (Figure 5-3A). This may have resulted from recombination events resulting in the loss of the recombinant gene,

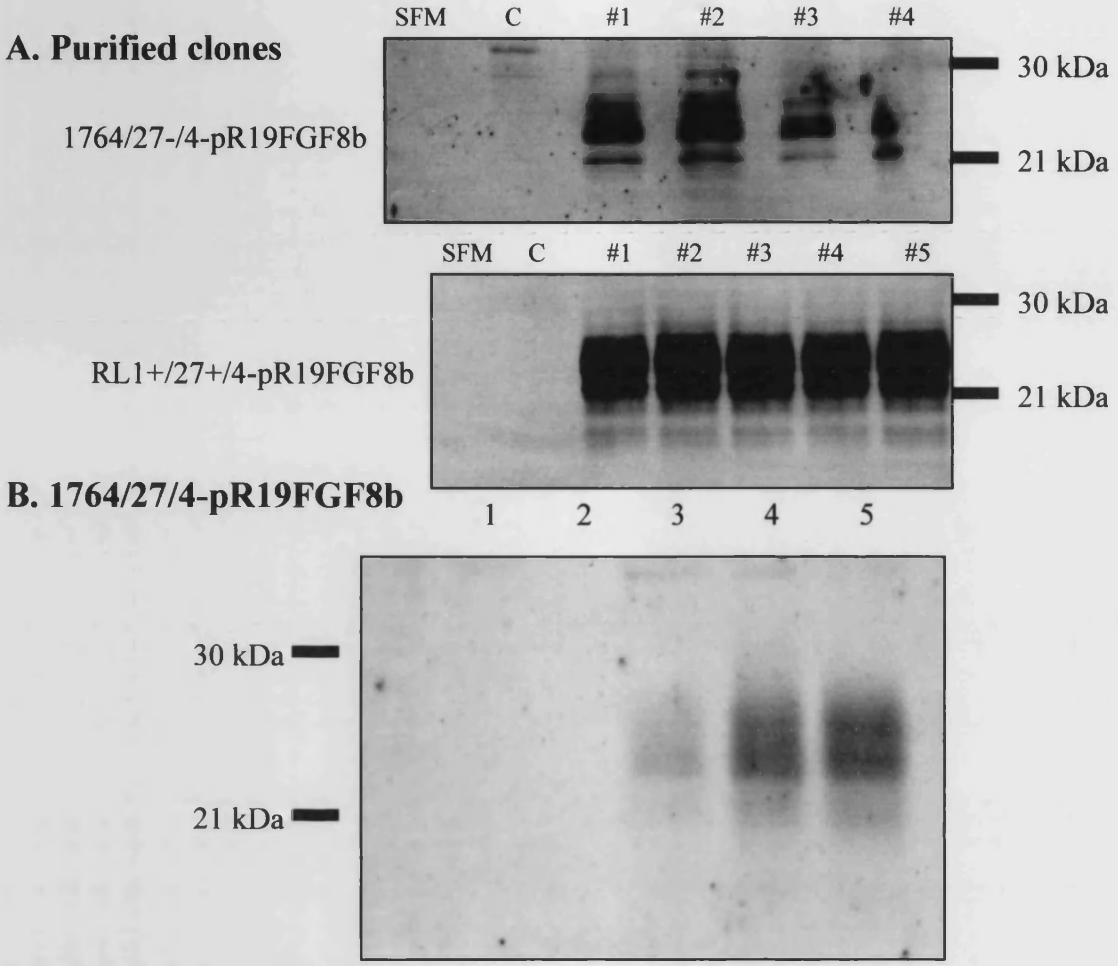
although this problem was not encountered in recombinant constructs based on the less disabled viral backbone. Positive clones were selected and propagated to produce high titre viral stocks.

To ensure that recombinant FGF8 and FGF2 were also expressed and secreted in non-complementing cells, BHK cells were infected with the different constructs. Two days post infection the cells were overlaid with serum free media and the supernatant collected at different time points. After concentration using size exclusion chromatographic mini-columns the proteins were separated by SDS-PAGE and immunoblotted for FGF8 and FGF2, respectively. As shown for the highly disabled constructs, FGF8b (Figure 5-2B) and FGF2 (Figure 5-3B) were expressed in these non-complementing cells and secreted into the supernatant.

western blotting for FGF8 showed a ladder of bands of different molecular weights around the expected molecular size of 23kDa (Dorkin et al., 1999) which is probably the result of multiple N-linked glycosylation events (Crossley and Martin, 1995). A very strong signal was also observed from cell-associated and supernatant samples from infected BHK cells for RL1+/27+/4-pR19FGF8bwpre (Figure 5-2C) confirming that a large amount of virally produced FGF8 was released from these cells.

Smaller amounts of FGF2 appeared to be secreted after infection of BHK cells with 1764/27-/4-/pR19FGF2. The protein ran on the SDS-PAGE at a molecular weight of about 19kDa which conforms to the reported protein size of 18-20 kDa for FGF2 (Brickman et al., 1995). Interestingly, unlike the other members of the FGF family, FGF2 lacks amino-terminal signal peptides and is apparently secreted by mechanisms other than the classical protein secretion pathway (Mignatti et al., 1992) and mainly remains cell-associated on the cell surface and within the extracellular matrix (Ornitz and Itoh, 2001). This was also observed in western blot analysis after infection of BHK cells with RL1+/27+/4-pR19FGF2wpre (Figure 5-3C). Virally produced FGF2 protein was strongly associated with the cell pellet but could also be detected in the supernatant. Some of the protein in the supernatant may also have resulted from the release from damaged cells.

Expression was also confirmed after infection of primary neural progenitor cells and neurospheres. Cortical, striatal and mesencephalic progenitor cells as well as striatal neurospheres were infected with RL1+/27+/4-pR19FGF8bwpre, RL1+/27+/4-pR19FGF2wpre or control virus (RL1+/27+/4-pR19GFPwpre). No endogenous expression of FGF8 (Figure 5-2C) or FGF2 (Figure 5-3C) was detectable from control samples. Significant levels of recombinant FGF8 and FGF2 were detected after infection with the respective constructs. Strong transgene expression was observed in neurospheres, while expression in neural progenitor cells was lower due to limited transduction efficiency in this cell system as discussed in chapter 3. However, considering the low concentration required for achieving biological effects (FGF8 was used before at concentrations of 50ng/ml and FGF2 at 20ng/ml), the virally produced proteins may be sufficient to study effects in primary neural progenitor cells.



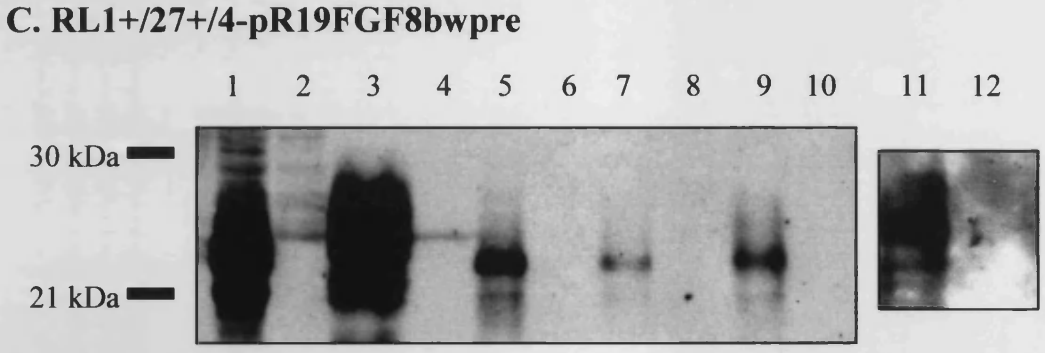


Figure 5-2: Western blot analysis confirmed expression of virally delivered FGF8b in various cell systems. (A) Purified clones were tested for their expression of FGF8 in virus growth complementing M49 cells. C=control virus (B) BHK cells were infected with (1) 1764/27-/4-pR19LacZ, (2) SFM, or (3) to (5) 1764/27-/4-pR19FGF8b at an m.o.i. of 5 and overlaid with SFM three days post infection. Supernatant of the infected BHK's was collected (3) 2h, (4) 6h and (5) 24h later, concentrated, the proteins separated on a 15% SDS-PAGE and blotted on a nitrocellulose membrane. (C) Expression after infection with RL1+/27+/4-pR19FGF8bwpre was also confirmed in (1) cell pellet of BHK cells, (3) collected supernatant of infected BHK's, (5) cortical neural progenitor cells, (7) striatal neural progenitor cells, (9) mesencephalic neural progenitor cells and (11) striatal neurospheres. (2), (4), (6), (8), (10) and (12) are the respective cells infected with 1764/27-/4-pR19GFPwpre where no FGF8 expression was detected.

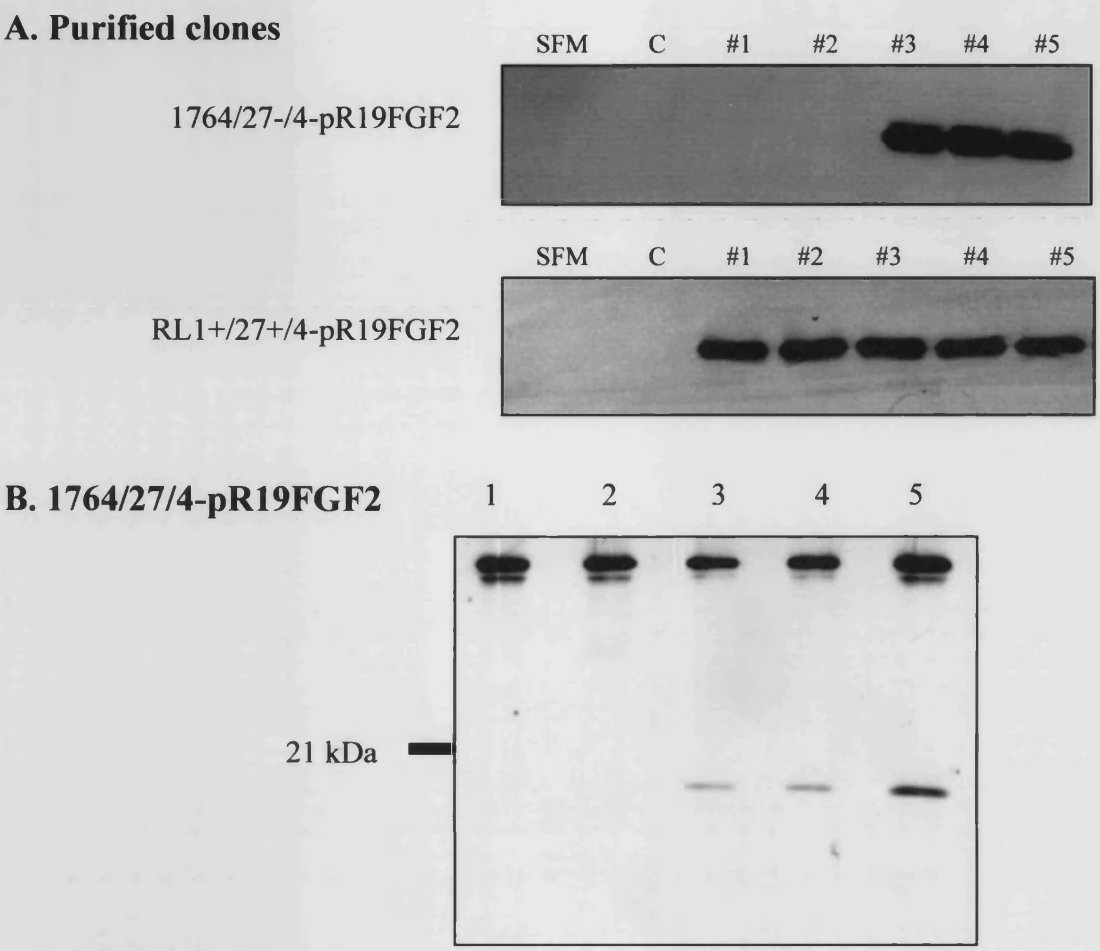


Figure 5-3: Western blot analysis confirmed expression of virally delivered FGF2 in various cell systems. (A) Different purified clones were tested for their expression of FGF2 in M49 cells. C=control virus (B) BHK cells were infected with (1) 1764/27-/4-pR19LacZ, (2) SFM, or (3) to (5) 1764/27-/4-pR19FGF2 at an m.o.i. of 5 and overlaid with SFM three days post infection. Supernatant of the infected BHK's was collected (3) 2h, (4) 6h and (5) 24h later, concentrated, the proteins separated on a 15% SDS-PAGE and blotted on a nitrocellulose membrane. (C) Expression after infection with RL1+/27+/4-pR19FGF2wpre was also confirmed in (1) cell pellet of BHK cells, (3) collected supernatant of infected BHK's, (5) cortical neural progenitor cells, (7) striatal neural progenitor cells, (9) mesencephalic neural progenitor cells and (11) striatal neurospheres. (2), (4), (6), (8), (10) and (12) are respective cells infected with 1764/27-/4-pR19GFPwpre where no FGF2 expression was detected.

5.3.2.2 Virally delivered FGF2 and FGF8 induce neurite outgrowth in PC12 cells

FGF2 and FGF8 not only have mitogenic effects as discussed in previous chapters, but can also induce neuronal differentiation in cultures of rat PC12 cells (Rydel and Greene, 1987; Tanaka et al., 2001). Neuronal differentiation is detectable by the formation of long and branched processes. This neurite outgrowth assay was used to confirm the bioactivity of virally produced FGF2 and FGF8 (Figure 5-4).

Infection of PC12 cells with the highly and less disabled viral constructs resulted in rather low gene delivery at m.o.i's between 1 to 30. In order to transduce about 50-70% of the cells an m.o.i. of about 300 was required as measured by GFP expression. Reporter gene expression using 1764/27-/4-pR19GFP was faint and difficult to document with the available system. However, even at these high multiplicities no cytotoxic effects compared to uninfected PC12 cells were observed.

PC12 cells infected with FGF2 and FGF8 expressing constructs began to evolve neurite processes three days post infection. On the other hand, positive controls containing recombinant proteins from the day of transduction had already formed long neurites at this time point. Most likely a threshold concentration of virally produced proteins was required to induce differentiation. Neural process formation continued until the experiment was terminated six days post infection. At this time point PC12 cells infected with viral constructs expressing FGF2 or FGF8 had long and branched neurite processes. Control cultures that were uninfected or transduced with reporter gene expressing vectors did not show significant signs of differentiation.

Quantification of the neurite outgrowth showed a significant increase in process formation for cultures infected with FGF8 or FGF2 expressing vectors ($p<0.001$ compared to controls). No statistical difference between virally and bacterially produced recombinant FGF8 and FGF2 was observed suggesting similar biological effects. Small statistical differences ($p<0.05$) between virally produced FGF8 and FGF2 and between the vector backbones may reflect differences in the expression level of the respective viral constructs.

Hence, the neurite outgrowth assay confirmed that virally produced FGF8 and FGF2 were bioactive and induced neuronal differentiation in the chosen system.

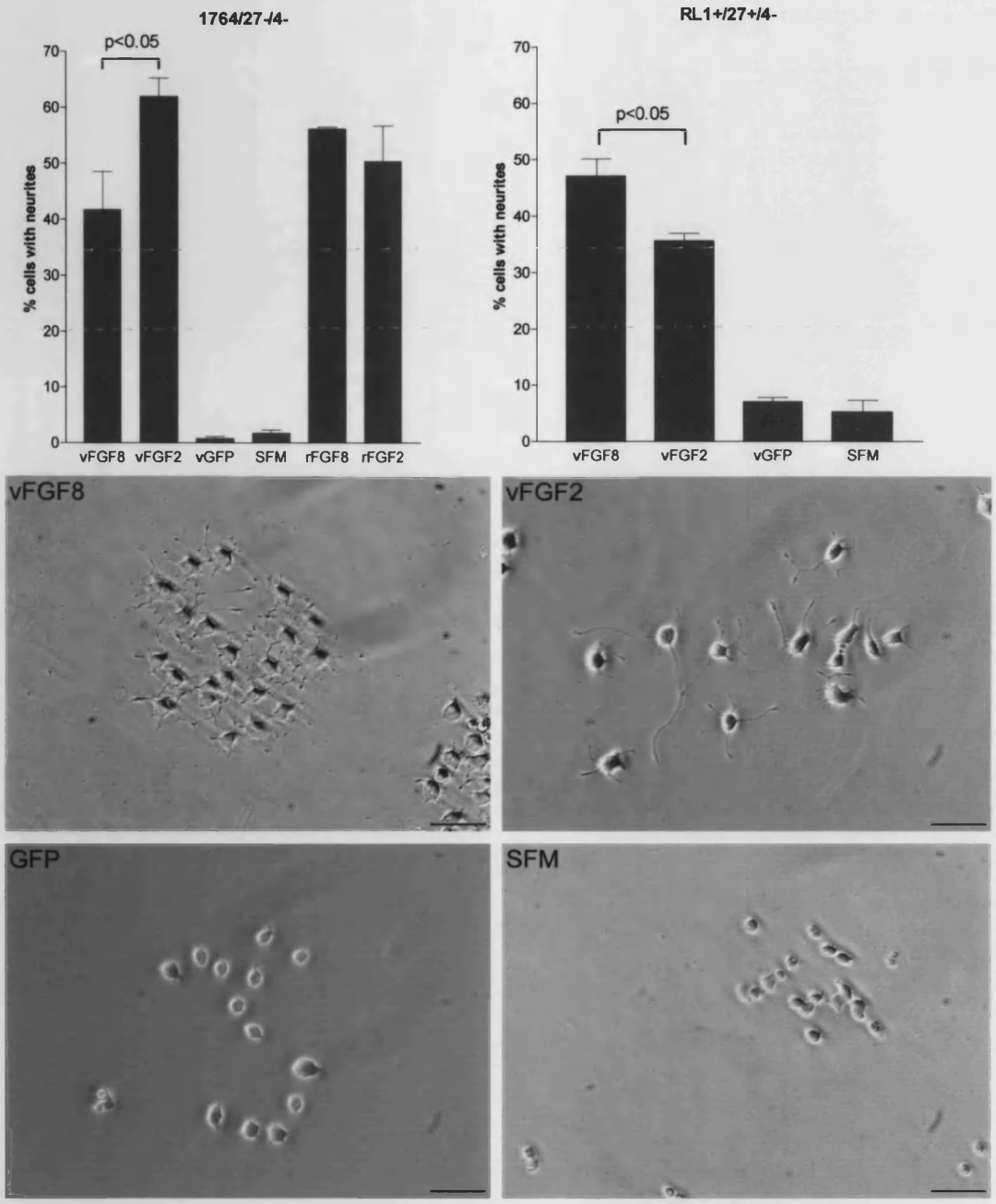


Figure 5-4: Virally delivered FGF2 and FGF8 induce neurite outgrowth in PC12 cells. PC12 cells were infected with 1764/27-/4-pR19FGF8b, 1764/27-/4-pR19FGF2 or RL1+/27+/4-pR19FGF8bwpre, RL1+/27+/4-pR19FGF2wpre. Negative controls were 1764/27-/4-pR19GFP, RL1+/27+/4-pR19GFPwpre and SFM. As positive controls recombinant FGF8 (50ng/ml) and FGF2 (5ng/ml) were added every other day from the day of infection. 6 days after infection the percentage of cells with processes longer than the cell diameter was counted. Data represent the means of cells with neurite processes as percentage of total cell number with SEM from three independent experiments. Virally delivered FGF8 (vFGF8) and FGF2 (vFGF2) significantly induced neurite outgrowth compared to GFP and SFM controls ($p<0.001$, n=3 experiments in One-way ANOVA). Lower statistical differences were detected between vFGF8 and vFGF2 ($p<0.05$; n=3 experiments in One-way ANOVA). The phase contrast images show the morphological differences resulting from infection with FGF8 and FGF2 expressing highly disabled constructs. No morphological changes were detected after transduction with GFP expressing control virus. Scale bar represents 50µm.

5.3.2.3 Virally delivered FGF2 and FGF8 have mitogenic effects on primary cortical and mesencephalic neural progenitor cells

The previous chapter has shown that FGF8 and FGF2 are mitogens for the expansion of neural precursor cells in neurosphere cultures. In chapter 3 the population of neural progenitor cells was characterized and it was found that at early time points these cultures contain a population of undifferentiated nestin positive cells. The population of undifferentiated cells decreased over time in culture due to differentiation into post-mitotic neural cell types. In order to establish if progenitor cultures can be maintained in a proliferative state, the potential mitogenic effects of virally delivered FGF8 and FGF2 were explored.

Neural progenitor cells derived from E14 cortex and mesencephalon were infected on 1 DIV with the less disabled constructs expressing either FGF8 or FGF2. Negative controls were infected with reporter gene expressing virus or SFM instead. To label the proliferating cells on 3-4 DIV progenitor cells were pulse labelled with BrdU (0.2μM) for 12 hours. Immunocytochemical staining for BrdU were performed to detect cells that have incorporated the thymidine substitute and hence, were characterized as cells with DNA synthesis. To quantify the effects on proliferation the number of BrdU positive cells as percentage of total cell number was determined (Figure 5-5).

Virally delivered FGF8 and FGF2 significantly increased the number of BrdU incorporating cortical ($p<0.01$ for all vs. SFM in One-way ANOVA; n=3 independent experiments) and mesencephalic ($p<0.05$ for FGF8 vs. SFM, $p<0.001$ for FGF2 vs. SFM in One-way ANOVA; n=3 independent experiments) progenitor cells. Surprisingly, in uninfected cortical cultures about 30% of the cells incorporated BrdU despite the lack of exogenous growth factors. However, after delivery of FGF8 and FGF2 about 83% of the cells incorporated BrdU. Proliferative effects were more prominent in cortical than mesencephalic progenitor cultures. In mesencephalic progenitor cultures only 10% of the control cells were BrdU positive, an amount that could be increased by viral delivery of FGF8 and FGF2 to 45% and 67% respectively.

Proliferative effects of FGF8 and FGF2 on progenitor cells were not only observed by BrdU incorporation but also demonstrated by an increased cell number in those cultures.

To ensure that BrdU incorporation resulted mainly from cell division, mitosis was inhibited in FGF2 and FGF8 infected cells by administration of the anti-mitotic drug cytosine-β-D-arabinofuranoside (Ara-C) (Figure 5-6). In the absence of Ara-C mitogenic effects of virally delivered FGF8 and FGF2 were observed as shown above. Addition of Ara-C completely abolished BrdU incorporation in cultures infected with control virus as well as with FGF8 or FGF2 expressing vectors. As Ara-C inhibits DNA synthesis (Kufe and Major, 1982), BrdU incorporation resulted most likely from cell division. By reversing the mitogenic effects of virally delivered FGF8 and FGF2 it was therefore confirmed that the expressed proteins did indeed induce proliferation of progenitor cells that would otherwise differentiate into post-mitotic neural cell types.

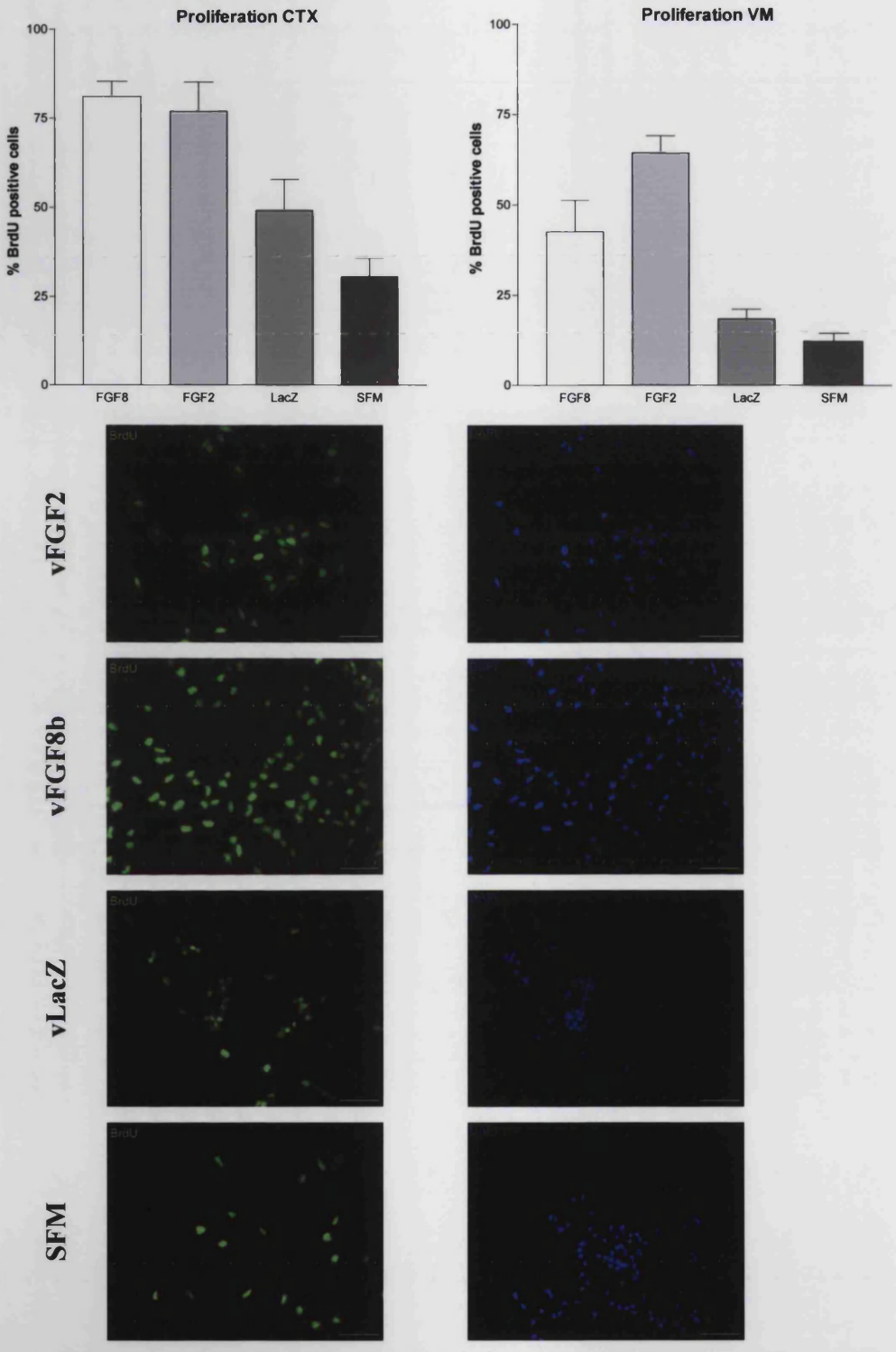


Figure 5-5: Virally delivered FGF8 and FGF2 increased BrdU incorporation in cortical and mesencephalic progenitor cells. Progenitor cells were infected with RL1+/27+/4- constructs expressing FGF8, FGF2 or as controls LacZ and SFM. Cells were pulse labelled with BrdU (0.2μM) for 12 hours three days after infection. Cells were fixed and stained for BrdU (Alexa 488, green) and cell nuclei stained with DAPI (blue). Data represent means of BrdU incorporated cells as percentage of total cells from three independent experiments. Virally delivered FGF8 and FGF2 significantly increase the number of BrdU positive cells in cortical ($p<0.01$ for FGF8 and FGF2 vs. SFM; $p<0.05$ for both vs. LacZ) and mesencephalic ($p<0.05$ for FGF8 vs. LacZ and SFM, $p<0.01$ for FGF2 vs. LacZ and $p<0.001$ for FGF2 vs. SFM) progenitor cells.

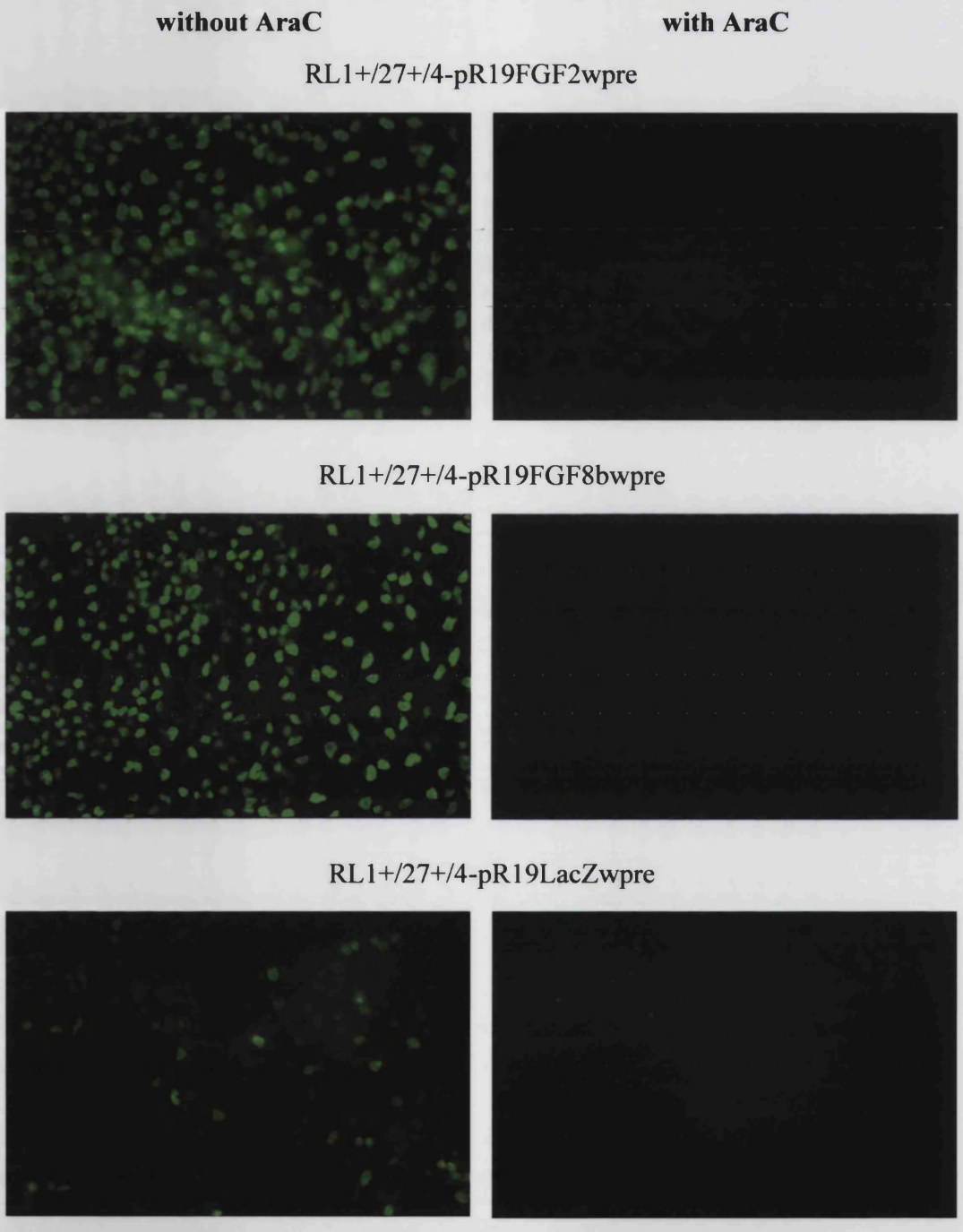


Figure 5-6: Treatment with cytosine-β-D-arabinoturanoside (Ara-C) abolished the mitogenic effects of FGF8 and FGF2. E14 cortical progenitor cells were infected on 1 DIV and maintained in Neuobasal™/B27 media. On 3 DIV cells were pulse labelled with BrdU (0.2μM) for 12 hours in the presence or absence of Ara-C (20μM). The cells were fixed and stained for BrdU (Alexa 488, green). In the absence of the anti-mitotic drug an increased number of progenitor cells incorporated BrdU when infected with FGF8 and FGF2 expressing vectors. In parallel cultures BrdU incorporation was almost completely inhibited when Ara-C was present. Hence, increased BrdU incorporation most likely resulted from proliferative effects of virally delivered FGF8 and FGF2.

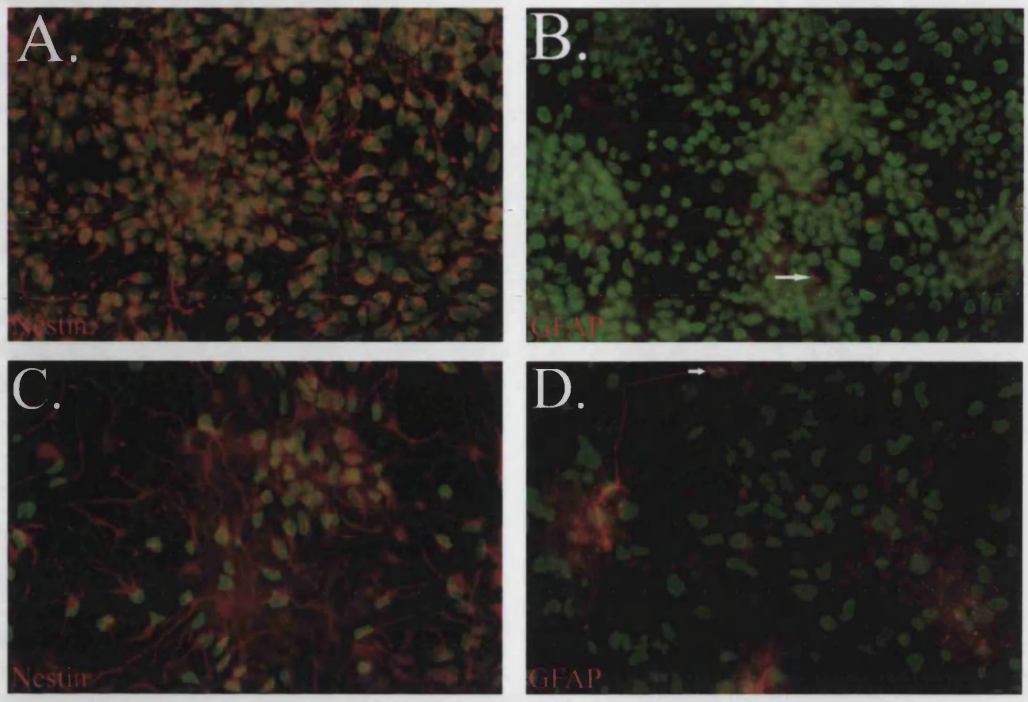
5.3.2.4 Characterization of proliferating cells

In order to establish the nature of the proliferating cells the antigenic character was identified. Therefore, cortical and mesencephalic progenitors infected with FGF8 and FGF2 expressing vectors were BrdU pulse labelled and maintained in differentiation conditions for another five days before fixation. Immunocytochemical double labelling for BrdU/nestin or BrdU/GFAP was performed (Figure 5-7).

Most of the BrdU positive cells double labelled with the neurofilament marker nestin. This was observed for cortical and mesencephalic progenitor cells infected with either FGF8 or FGF2 expressing viruses. None of the cultures contained a significant number of GFAP positive cells confirming that the dividing cells did not represent astrocytes. This is in agreement with results obtained in chapter 3 showing that the culture conditions do not support proliferation or differentiation of astrocytes.

Hence, virally delivered growth factors maintained primary neural progenitor cells in a proliferative and undifferentiated fate. As identified in chapter 3, neural progenitor cells undergo differentiation into post-mitotic neural cell types in the absence of additional growth factors accompanied by a decrease in the number of nestin positive cells. Here it was demonstrated that this differentiation pathway could be overcome by virally delivered FGF8 and FGF2 that maintain the precursor cell population over an extended time *in vitro*. It has yet to be confirmed if the generated cell population remained susceptible to other inductive signals that might allow their guided differentiation into specific neural cell types. As previously discussed, neural progenitor cultures have a very narrow time frame during which they remain plastic to alteration of their cell fate. By inhibiting early differentiation the progenitor cultures have therefore become more useful for testing other factors involving their conversion into a dopaminergic phenotype.

RL1+/27+/4-pR19FGF8bwpre



RL1+/27+/4-pR19FGF2wpre

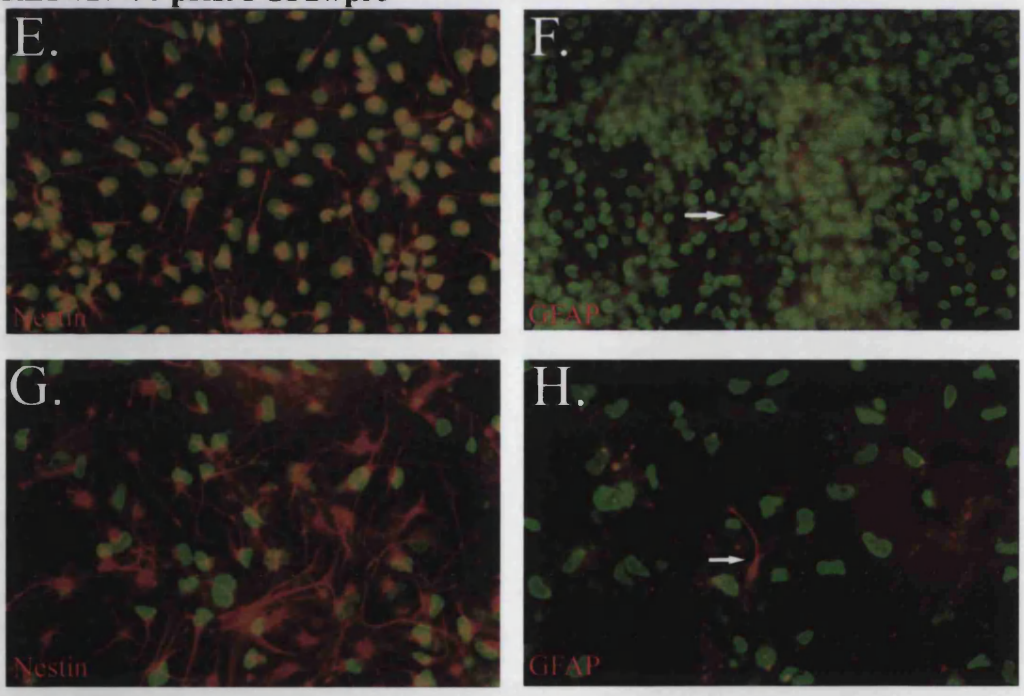


Figure 5-7: Characterization of progenitor cells stimulated by virally delivered FGF8 and FGF2. Cortical (A, B, E, F) and mesencephalic (C, D, G, H) progenitor cells were infected with the less disabled viral constructs expressing FGF8 or FGF2. Five days post transduction cells were fixed and double stained for BrdU (Alexa 488, green) and nestin (Alexa 546, red) or BrdU and GFAP (Alexa 546, red). Virtually all BrdU positive cells co-labelled with nestin. Only a small number of GFAP positive astrocytes (arrows) was detected in the cultures. 40x magnification.

5.3.2.5 Virally delivered FGF8 increases the number of TH+ve neurons in mesencephalic progenitor cultures

In the previous chapter it was shown that recombinant FGF8b exerts neurotrophic effects on primary post-mitotic dopamine neuroblasts and dopaminergic neurons grown as neurosphere cultures. In order to establish if similar effects could also be accomplished in primary mesencephalic cultures, the effects of virally delivered FGF8 in this cell system were studied.

E14 ventral mesencephalic progenitor cells were infected with the less disabled vectors expressing FGF8b or FGF2 on 1 DIV. As controls parallel cultures were transduced with LacZ expressing virus or SFM only. In order to label newly generated dopaminergic neurons the cultures were pulse labelled with BrdU (0.2μM) for 12 hours on 3-4 DIV. Cells were fixed five days post transduction and stained for BrdU and TH. The number of TH+ve cells was determined as the percentage of total cell number from 20 different fields and the means calculated from three independent experiments (Figure 5-8).

Virally delivered FGF8 significantly increased the number of TH positive cells in mesencephalic progenitor cultures compared to cultures infected with FGF2 expressing virus or controls ($p<0.05$ for FGF8 vs. all other treatments using One-way ANOVA, n=3 experiments). However, only an insignificant number of BrdU/TH double labelled cells was detected in either FGF8 infected progenitor cultures or any of the parallel treatments. This suggests that during the BrdU pulse (3-4 DIV) no cell division of dopamine neuroblasts occured in FGF8 cultures and hence, the increase of TH expressing cells is not the result of *de novo* generated dopaminergic neurons or an extended proliferation of dopamine neuroblasts. As discussed in the previous chapter the increased number is most likely due to trophic effects of virally delivered FGF8 on post-mitotic dopaminergic neurons.

Although an increased cell proliferation was detected in FGF2 infected progenitor cultures, only very few TH positive neurons were double labelled with BrdU

demonstrating that FGF2 did not extend the time of proliferation of dopamine neuroblasts. Other groups have previously reported that recombinant FGF2 can extend such dopamine proliferation and delay differentiation of the dopamine neuroblast (Bouvier and Mytilineou, 1995). However, Bouvier and Mytilineou used E12 mesencephalic progenitor cultures while the study here used E14 ventral mesencephalon. Different embryonic ages used in the studies may explain the discrepancy.

Thus, the increased precursor proliferation did not correlate with enhanced dopamine neuron expansion or differentiation.

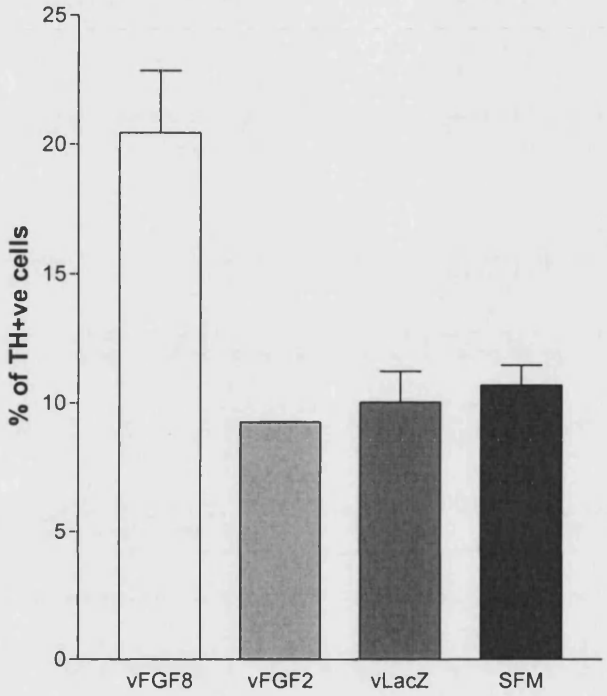


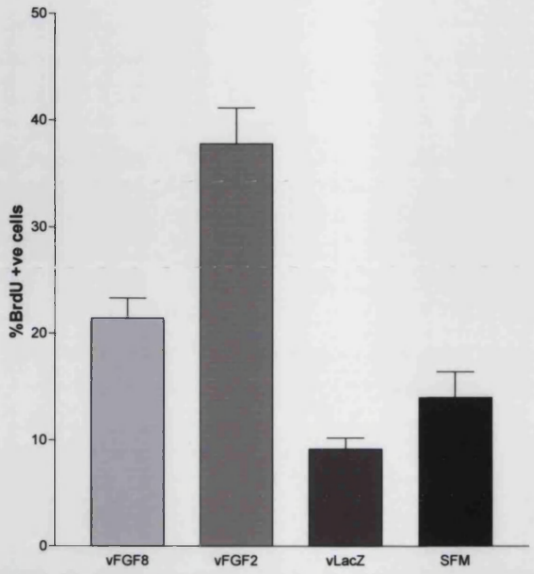
Figure 5-8: Virally delivered FGF8 increases the number of dopaminergic neurons in mesencephalic progenitor cultures. E14 ventral mesencephalic progenitor cells were infected with SFM or RL1+/27+/4- vectors expressing FGF8, FGF2 and LacZ on 1DIV. The number of TH positive cells as percentage of total cell numbers was determined from 20 different fields on 6 DIV. The data represent the means from three independent experiments. One-way ANOVA with Tukey's post-hoc analysis revealed a significant increased number of TH positive cells in cultures that were infected with FGF8 expressing vectors ($p<0.05$ vs. all other groups, n=3 experiments).

5.3.2.6 Effects of virally delivered FGF2 and FGF8 in neurospheres

Mitogenic effects of virally delivered FGF8 and FGF2 were tested in a BrdU incorporation assay after transduction of mesencephalic neurospheres (Figure 5-9). Increased cell proliferation was confirmed after transduction with viral vectors expressing FGF8 and FGF2. As shown for recombinant FGF8b in chapter 4, virally delivered FGF8 also induced proliferation of mesencephalic precursor cells ($p<0.01$ for vFGF8 vs. vLacZ in One-Way ANOVA, n=3 experiments). The rather small proliferative effect observed for virally delivered FGF8 was most likely due to the absence of heparin in the culture conditions. As discussed before, the mitogenic activity of FGF8 strongly depends on the presence of heparin. However, heparin inhibits viral transduction and was therefore omitted in this experiment. As FGF2 is a potent mitogen even in the absence of heparin, significantly more mesencephalic precursor cells responded after transduction with viral vectors expressing FGF2.

Interestingly, it was observed that dissociation of FGF8 infected neurospheres into single cells and maintenance under differentiation conditions gave rise to more GalC positive oligodendrocytes than FGF2 infected precursors or controls (Figure 5-9B). This observation corresponds to results from the previous chapter showing a shift towards the generation of more oligodendrocytes in FGF8 expanded mesencephalic neurosphere cultures. It has previously been shown that FGF8 mainly transduces via FGFR3IIIc in neural precursor cells (Hajihosseini and Dickson, 1999) and its activation also plays crucial roles in oligodendrocyte development (Oh et al., 2003b). Thus, it is possible that virally delivered FGF8 did indeed support development of oligodendroglial progenitor cells. However, as this observation was not relevant to the study, such effects remained unquantified, although they could be of interest in the future.

A.



B.

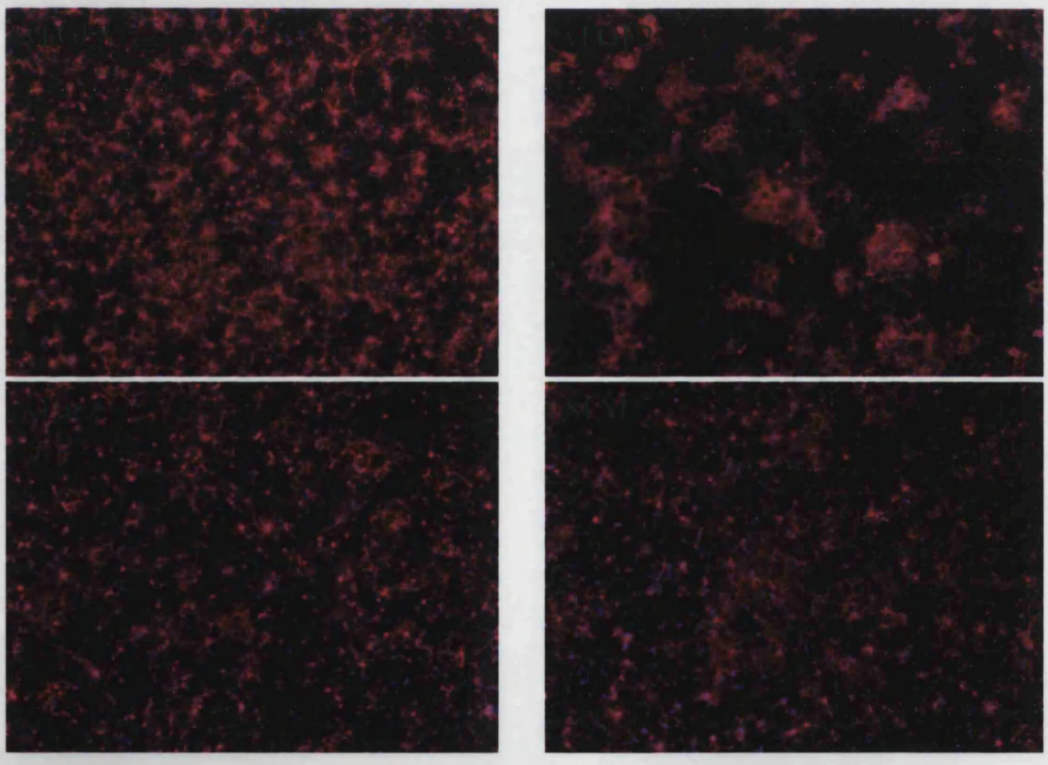


Figure 5-9: Effects of virally delivered FGF8 and FGF2 in mesencephalic neurospheres. (A) E14 mesencephalic neurospheres were infected with SFM or highly disabled backbone 1764/27-/4-expressing FGF8, FGF2 or LacZ. Three days post transduction neurospheres were pulse labelled with BrdU (0.2μM) for 12 hours, dissociated and plated on substrate coated glass cover slips. Cells were fixed after attachment and stained for BrdU. The number of BrdU positive cells as percentage of total cell number was determined from 20 different fields. Data represent means with SEM from three independent experiments. Significant increase of cell proliferation was detected for vFGF8 ($p<0.01$ vs. LacZ and $p<0.05$ vs. SFM) and vFGF2 ($p<0.001$ vs. LacZ and SFM). (B) Neurospheres infected and dissociated as described above were stained for GalC (Alexa 546, red) and cell nuclei stained with Hoechst (blue). A high number of oligodendrocytes was detected in FGF8 infected ^{mes}NS. 10x magnification.

5.3.3 The effects of virally delivered ShhN in primary neuronal cultures and neurospheres

5.3.3.1 Construction of HSV-1 vectors expressing ShhN

Conversion of Shh into an active form requires multiple posttranslational modification steps and distinct secretion pathways (Figure 5-10A). Shh (45kDa) is produced as an inactive precursor protein which converts itself by autocatalytic internal cleavage (Porter et al., 1995; Bumcrot et al., 1995; Lee, 1998) into an active aminoterminal molecule, the 19kDa NH_2-terminal domain of sonic hedgehog (ShhN, residue 25-198) mediating all signaling activities in vertebrates and invertebrates (reviewed by Hammerschmidt, 1997), and a 25kDa C-terminal protein (ShhC) that possesses protease activity (Porter et al., 1995; Porter et al., 1996a). ShhN is further modified by cholesterol attachment at its COOH-terminal (Porter et al., 1996a; Porter et al., 1996b) and addition of a palmitolyl group to the N-terminal of the processed ShhN (Porter et al., 1996a; Pepinsky et al., 1998; Chamoun et al., 2001). These posttranslational modifications play critical roles in Shh signaling and increase the inductive potency of ShhN (Marti and Bovolenta, 2002). Processed ShhN can remain in the cell membrane in bound form, with affinity to extracellular matrix glycoproteins such as vitronectin enhancing Shh activity (Pons and Marti, 2000), or secreted in a soluble form (Porter et al., 1996a). Secreted ShhN can form disulfide bonds that also affect its biological activity (Saeki et al., 2000) and due to its lipophilic modifications soluble ShhN can multimerize with itself (Zeng et al., 2001). The distinct biological functions reported for ShhN as morphogen, mitogen, survival factor or axonal guidance factor (reviewed by Marti and Bovolenta, 2002) may likely resemble those different forms available during development and adulthood.

Viral vectors were generated expressing the bioactive aminoterminal part of rat Shh (ShhN). Therefore, PCR primers were designed amplifying the openreading frame to produce a protein encompassing the first 198 amino acids of rat ShhN (Gene Bank accession number AF162915) by introducing an artifical stop codon after nucleotides

908. The sequence of the generated cDNA including the introduced stop codon was confirmed by sequencing and is attached in the appendix. After subcloning into the pR19 expression cassettes the generated plasmids, pGEM-5pR19ShhNLAT and pGEM-5pR19ShhNwpreLAT, were co-transfected with viral DNA from 1764/27-/4-pR19GFPLAT or RL1+/27+/4-pR19GFPwpreLAT, respectively. Recombinant plaques were visualised under the fluorescent microscope by the absence of GFP expression, purified and were named 1764/27-/4-pR19ShhN or RL1+/27+/4-pR19ShhNwpre, respectively. Expression of ShhN from different clones was confirmed by western blotting on virus growth complementing 27/12/M:4 cells as described above for FGF8 and FGF2 expressing vectors. Selected clones were propagated to high titer stocks.

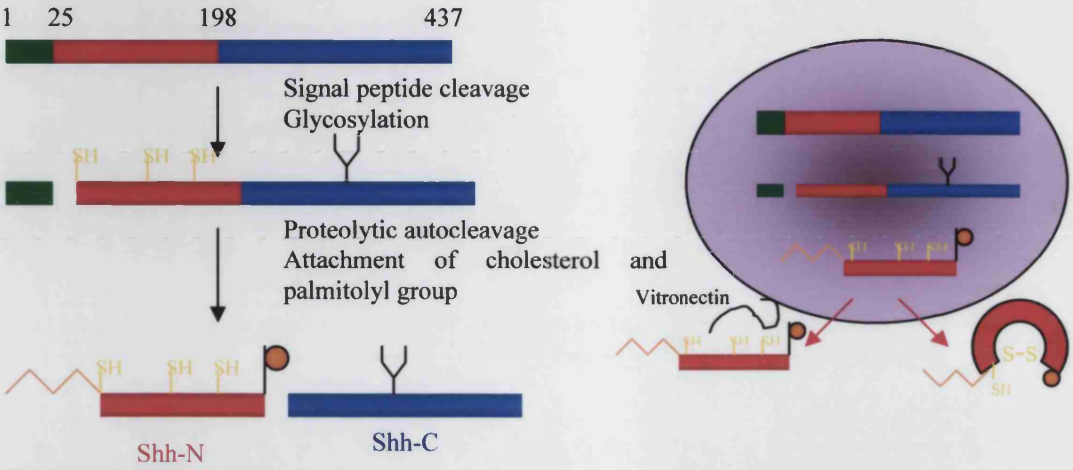
Expression and secretion of virally delivered ShhN was tested on non-complementing BHK cells (Figure 5-10B) after infection with ShhN expressing constructs. As described above, the expression of ShhN was analysed from cell-associated and supernatant samples on denaturating SDS-PAGE. Western blotting confirmed that ShhN was expressed in BHK cells and released into the supernatant. Soluble ShhN accumulated in the supernatant as increased amounts of protein were detected over time. The virally produced ShhN ran at about 21 kDa, slightly larger than the predicted molecular weight of 19kDa. However, this was in agreement with observations from other groups showing a molecular weight of 20 to 24 kDa on SDS PAGE under reducing conditions (Katsuura et al., 1999; Saeki et al., 2000).

The western blotting revealed an interesting finding. Supernatant samples contained two additional bands of ShhN protein, one that ran slightly faster than the main band and another that ran significantly slower at about 44 kDa. These additional bands were only found for secreted ShhN but not in samples generated from the respective cell pellet suggesting that ShhN must have been further modified after secretion. The smaller protein band was detected at all time points taken, while the larger protein band only appeared at the latest time point when a large amount of recombinant protein had accumulated in the supernatant. The faster running band was unlikely the result from cholesterol addition as it was not detected in cell-associated samples. It has been reported that soluble ShhN can form disulfide bonds between the three

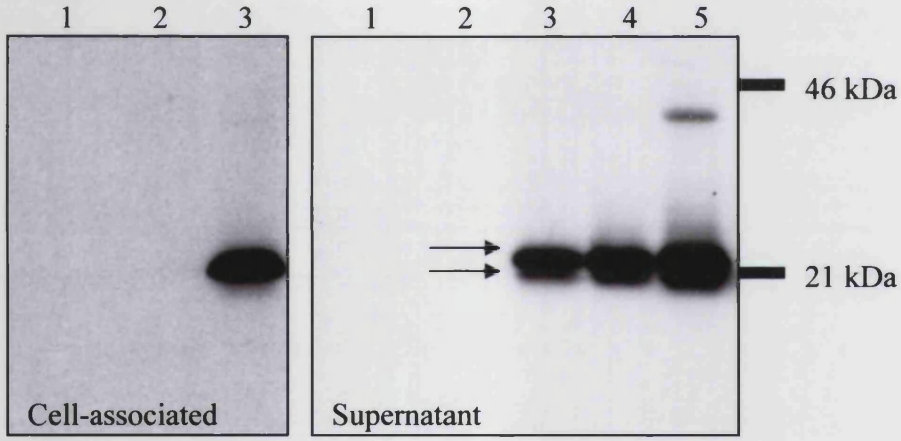
cysteine residues (Cys-25, Cys-103, Cys-184) within its N-terminal form that result in a pattern of shifted bands (Saeki et al., 2000). Although denaturing conditions were used, insufficient reduction may explain the small amount of this faster migrating protein band. The larger band is most likely due to multimerisation of ShhN as has previously been shown for soluble ShhN (Zeng et al., 2001). Despite its two lipophilic modifications ShhN is soluble in an aqueous environment and this is most likely to be possible by binding additional proteins to bury its hydrophobic moieties. This could also explain why agglomerates of ShhN were only found at high protein concentrations.

The expression of ShhN from the less disabled RL1+/27+/4- constructs was also confirmed in non-complementing cell systems. ShhN was expressed and secreted by RL1+/27+/4-pR19ShhNwpre in BHK cells showing the same additional bands in samples generated from concentrated supernatant as mentioned above. Infection of primary ventral mesencephalic neural progenitor cells with RL1+/27+/4-pR19ShhNwpre also confirmed expression while no detectable ShhN was found in control infected cultures. Strong expression of ShhN was also demonstrated in mesencephalic neurospheres after infection with the construct.

A. Shh processing



B. 1764/27/4-pR19ShhN



C. RL1+/27+/4-pR19ShhNwpre

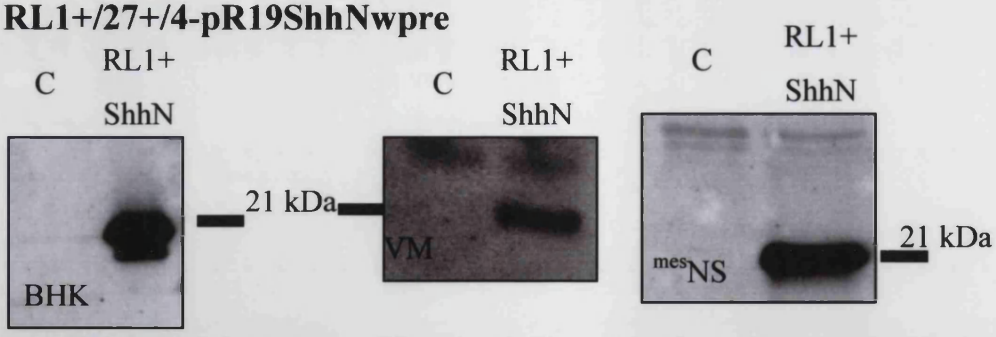


Figure 5-10: Western blot analysis confirmed expression of virally delivered ShhN in various cell systems. (A) Shh processing as described in text. (B) BHK cells were infected with (1) 1764/27-/4-pR19LacZ, (2) SFM, or (3) to (5) 1764/27-/4-pR19ShhN at an m.o.i. of 5 and overlaid with SFM three days post infection. Supernatant of the infected BHK's was collected (3) 2h, (4) 6h and (5) 24h later. ShhN was found to be expressed in the cell pellet and supernatant. Western blotting of supernatant protein contained two additional bands. The smaller band (arrow) represents most likely secreted forms of protein that formed disulfide bonds, while the larger band (45kDa) contained agglomerated multimers. (C) Expression after infection with RL1+/27+/4-pR19ShhNwpre was also confirmed in the supernatant of infected BHK's, mesencephalic neural progenitor cells and mesencephalic neurospheres. C=infection with control virus RL1+/27+/4-pR19GFPwpre.

5.3.3.2 Virally delivered ShhN induces differentiation of C3H10T1/2 fibroblasts into osteoblasts

Bioactivity of produced ShhN has often been assessed by the activity of alkaline phosphotase (AP) in the mouse fibroblast cell line C3H10T1/2 (Kinto et al., 1997; Howard et al., 1998; Williams et al., 1999; Saeki et al., 2000; Zeng et al., 2001). The cell line provides a simple system for determining ShhN function without the complication of having to work with primary cell cultures or organ explants. C3H10T1/2 cells are ShhN-responsive as assessed by induction of alkaline phosphatase (AP), a marker of mature, differentiated osteoblasts (Williams et al., 1999).

C3H10T1/2 cells were infected with ShhN or GFP expressing viral constructs based on 1764/27-/4- or RL1+27+4- backbone and differentiated in reduced serum conditions for five days. The AP assay was performed resulting in a deep purple stain in cells with AP activity (Figure 5-11).

Strong AP activity was determined in C3H10T1/2 cells with ShhN expressing constructs, while no activity was observed in the control cultures. The purple precipitate was mainly localised throughout the cytoplasm of the cells and significantly less abundant in the nucleus.

The experiment has confirmed that virally delivered ShhN is bioactive by inducing differentiation of C3H10T1/2 cells into osteoblasts, characterized by the activity of alkaline phosphatase.

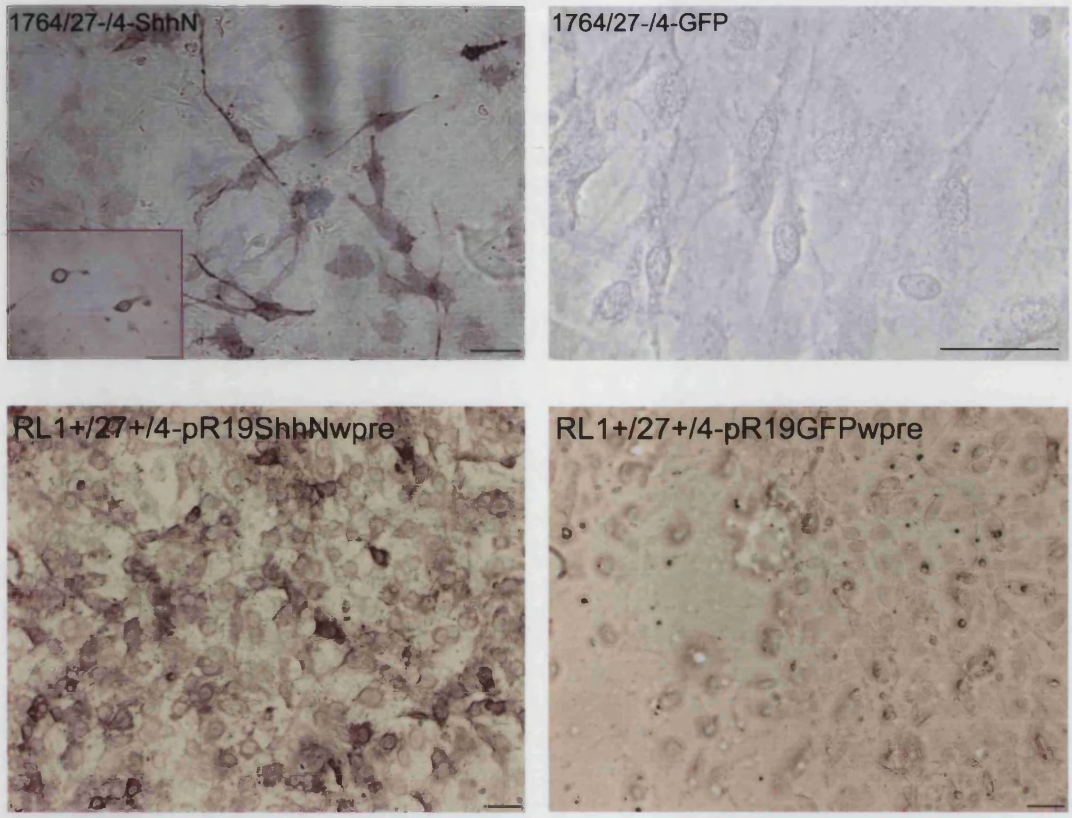


Figure 5-11: Assay for alkaline phosphatase (AP) confirming bioactivity of virally delivered ShhN. Mouse fibroblast C3H10T1/2 cells were infected at an m.o.i. of 3 with 1764/27-/4-pR19ShhN, RL1+/27+/4-pR19ShhNwpre or the respective control viruses expressing GFP. Cells were maintained for five days in DMEM/3% FCS and AP activity determined using a histochemical AP kit (Sigma). AP activity is visible as a deep purple precipitate. Cells infected with ShhN expressing vectors showed AP activity, while no activity was found in control cultures. Inset shows localisation of the stain predominantly in the cytoplasm of ShhN treated cells. Consequently, virally delivered ShhN did induce differentiation of C3H10T1/2 cells into AP positive osteoblast and thus, is bioactive. Scale bar represents 50µm.

5.3.3.3 Virally delivered ShhN has no significant effects in primary neuronal cultures and neurospheres

The previous chapter has shown that recombinant ShhN is neurotrophic for midbrain dopaminergic neurons cultured as neurospheres when present during the expansion phase. The work presented here tried to investigate if virally delivered ShhN exerts similar effects in primary midbrain dopaminergic neurons.

E14 midbrain dopaminergic neurons were infected with either SFM or less disabled vectors expressing ShhN or LacZ. Five days post-transduction the number of dopaminergic neurons was determined (Figure 5-12A) for the different treatments. No significant difference in the number of TH expressing cells was determined in the presence of virally expressed ShhN. This is surprising and opposed to findings of other groups clearly showing trophic effects of ShhN on TH survival of primary midbrain dopaminergic neurons generated from the same gestational age (Miao et al., 1997b). That study showed that ShhN concentrations of above 25ng/ml significantly delayed dopaminergic cell death and rescued an increased amount of TH expressing cells after one week in culture. However, lower concentrations of ShhN did not have this trophic effect and apparently a threshold of protein needs to be present to facilitate the effect. For this reason it is possible that the expression levels from the vector were not sufficient to achieve the desired survival effect on dopaminergic neurons.

As potential roles of ShhN on dopamine development from neural precursor cells were studied in the previous chapter, it was now determined if virally delivered ShhN alters the cell fate of precursor cells grown as neurospheres. Since the role of Shh on oligodendrocyte specification has been well established (Marti and Bovolenta, 2002), the amount of O4 positive cells was quantified after infection of cortical neurospheres (Figure 5-12B). No significant differences in the percentage of O4 positive oligodendrocytes could be detected compared to controls. The total cell number also seemed unaffected by virally delivered ShhN suggesting that it did not have obvious mitogenic effects on neural precursor cells as has been found for embryonic

development (Britto et al., 2000), and also reported for adult hippocampal neural precursor cells (Lai et al., 2002). The previous chapter also suggested that recombinant ShhN had no significant proliferative effects on forebrain or midbrain neural precursor cells under the culture conditions used. No apparent changes in the amount of GFAP positive astrocytes or TuJ1 positive neurons were observed when ShhN was delivered to mesencephalic neurospheres (Figure 5-12).

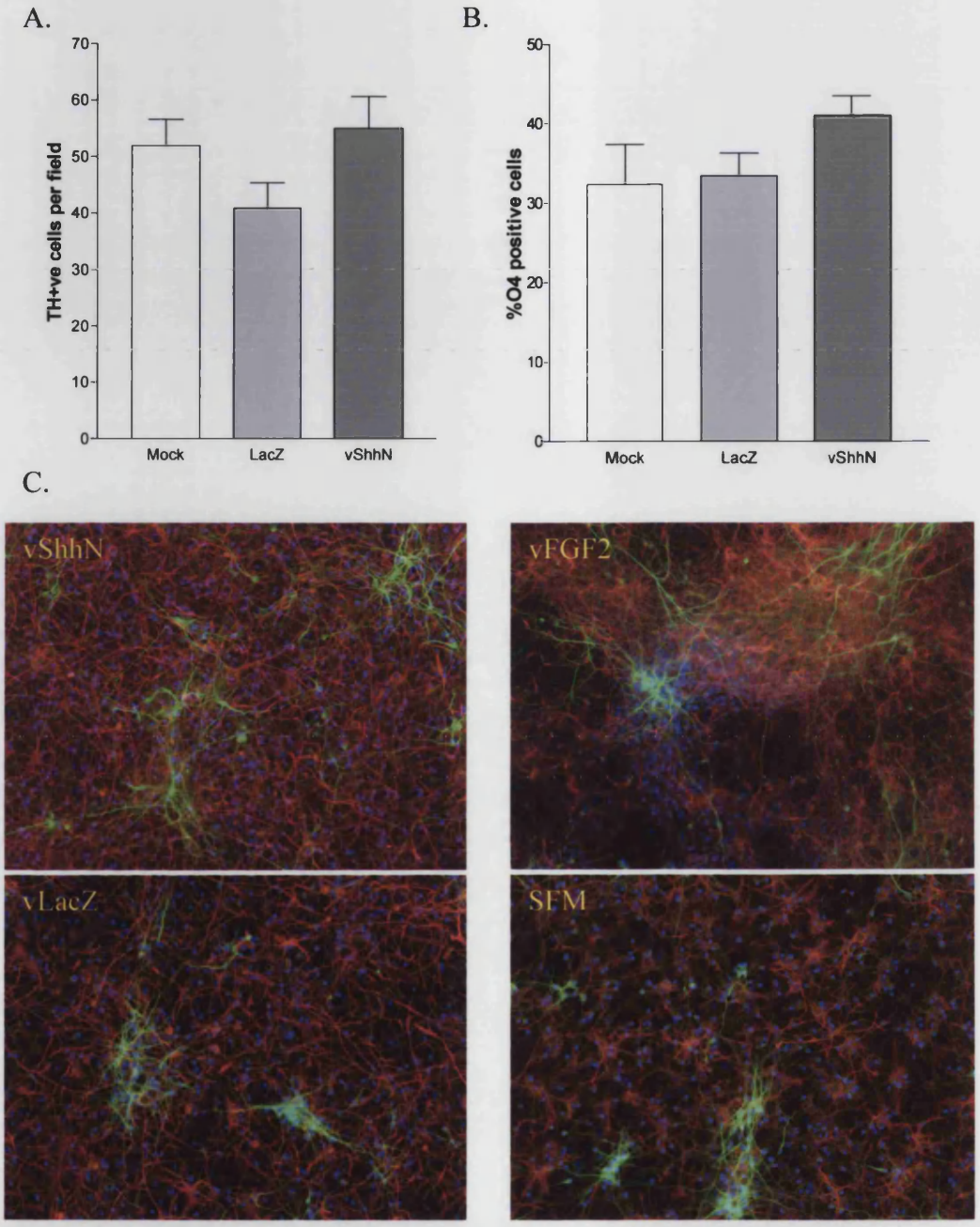


Figure 5-12: Effects of virally delivered ShhN in neural progenitor cells and neurospheres. (A) TH survival in primary E14 mesencephalic cultures after infection with RL1+/27+/4-pR19ShhN or the respective control virus expressing LacZ. No significance differences in the number of TH positive cells were noted in One-way ANOVA. (B) Cortical neurospheres were infected with RL1+/27+/4-pR19ShhN and differentiated for five days in the absence of growth factors. The number of O4 positive cells was determined in a migration assay. One-way ANOVA did not reveal significant differences in the number of O4 positive cells ($p>0.05$). (C) Infection of ^{mes}NS with 1764/27-/4- based vectors expressing ShhN, FGF2 or LacZ. Infected neurospheres were dissociated after infection, plated on poly-L-lysine/Laminin coated glass coverslips and differentiated for five days. Immunocytochemical staining for GFAP (Alexa 546, red) and TuJ (Alexa 488, green) did not show obvious differences in the amount of astrocytes or neurons generated after infection with viral vectors expressing ShhN, FGF2 or LacZ. Cell nuclei were stained for DAPI (blue).

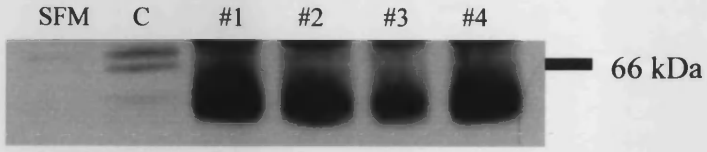
5.3.4 The effects of virally delivered Nurr1 in primary neuronal cultures and neurospheres

5.3.4.1 Construction of HSV-1 vectors expressing Nurr1

The gene encoding for Nurr1 was amplified from rat cDNA by PCR and subcloned into the viral expression cassettes generating the plasmid pGEM-5pR19Nurr1LAT or pGEM-5pR19Nurr1wpreLAT. Recombinant viruses were generated by insertion of the respective cassettes into the LAT region of highly disabled vectors, generating 1764/27-/4-pR19Nurr1, or the less disabled backbone, creating RL1+/27+/4-pR19Nurr1wpre.

The expression of Nurr1 was confirmed by western blot analysis (Figure 5-13). The Nurr1 protein ran on 15%SDS PAGE with an approximate molecular weight of 66kDa which is in agreement with the predicted molecular weight (Law et al., 1992). As expected Nurr1 proteins remained cell-associated and were not secreted into the supernatant of infected non-complementing BHK cells. Nurr1 expression was also confirmed in neurosphere cultures derived from mesencephalic, cortical or striatal regions after transduction with RL1+/27+/4-pR19Nurr1wpre. Control infections did not express Nurr1.

A. Purified clones



B. Non-complementing cell systems

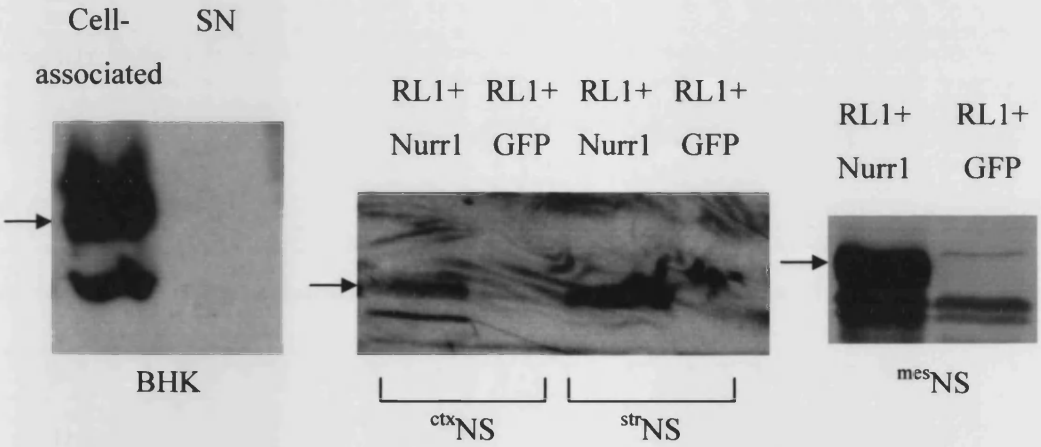


Figure 5-13: Western blot analysis confirmed expression of virally delivered Nurr1 in various cell systems after infection with RL1+/27+/4-pR19Nurr1wpre. (A) Purified clones of RL1+/27+/4-pR19Nurr1wpre were tested for their expression of Nurr1 in virus growth complementing M49 cells. C=control virus. (B) Infection of BHK cells detected Nurr1 protein exclusively in the cell pellet but not in the supernatant. Expression could also be confirmed after infection of cortical, striatal and mesencephalic neurospheres. Arrows indicate protein band of Nurr1.

5.3.4.2 Nurr1 does not induce TH expression in primary neuronal cultures

As characterized by immunogenic markers in chapter 3 neural progenitor cells derived from E14 cortex and striatum do not express TH. It has previously been shown that retrovirally delivered Nurr1 could induce TH expression in hippocampal derived progenitor cells independent of proliferation, survival or differentiation effects, but most likely due to direct activation of the *TH* gene by binding to *cis*-acting response elements within the TH promoter (Sakurada et al., 1999). Indeed it has been confirmed that three binding sites are responsible for induction of TH expression by Nurr1, but that TH activation remains cell type dependent (Kim et al., 2003b). This seems to be in agreement with earlier studies demonstrating that although Nurr1 is sufficient to induce TH expression in a neural stem cell line, other factors most likely secreted by co-cultured type 1 astrocytes are required for this function (Wagner et al., 1999). In order to establish if Nurr1 can induce ectopic TH expression the effects of virally delivered Nurr1 in primary neural progenitor cultures were examined.

Here neural progenitors from cortex, striatum and mesencephalon were used at early stages as they previously had been identified as containing undifferentiated nestin positive cells. Progenitor cells were infected on 1 DIV with the less disabled construct expressing Nurr1 and fixed five days post transduction. Immunocytochemical staining for TH was performed and the number of TH positive cells determined for mesencephalic cultures (Figure 5-14).

Neither control cultures nor Nurr1 infected cortical or striatal progenitor cells contained any TH expressing cells. Thus, virally delivered Nurr1 did not induce any TH expression in these cell types under the chosen conditions. No evident changes in the number of TuJ1 positive neurons were observed. No significant number of GFAP positive astrocytes was detected in any of the cultures which is in agreement with previous findings discussed in chapter 3.

Quantification of TH positive cells in mesencephalic cultures also did not reveal a significant increase in the number of dopaminergic neurons in Nurr1 infected treatments compared to controls.

The experiment has therefore shown that virally delivered Nurr1 does not result in a gain-of-function, i.e. TH expression, in neural progenitor cultures. As under the chosen conditions these cultures are neuron enriched and contain virtually no astrocytes, other factors that are required for the function of Nurr1 may be missing under these culture conditions. For this reason, Nurr1 mediated TH induction was next studied in a culture system that contains a large number of astrocytes and oligodendrocytes, i.e. neurospheres.

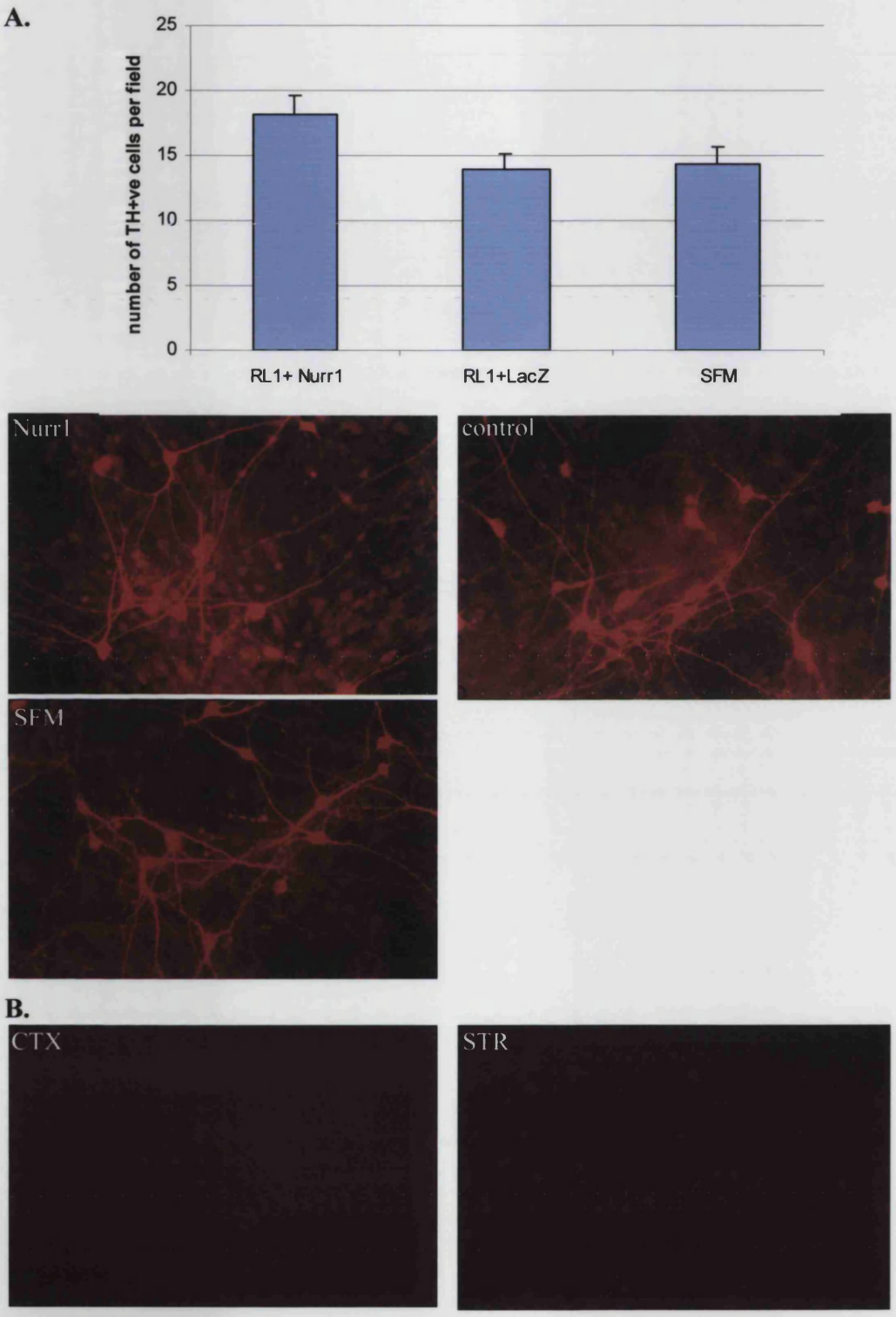


Figure 5-14: Effects of virally delivered Nurr1 in neural progenitor cultures. E14 cortical, striatal and mesencephalic progenitors were infected with RL1+/27+/4-pR19Nurr1 or the LacZ version, respectively. Five days post transduction cells were fixed and stained for TH (Alexa 546, red). (A) Quantification of the number of TH positive cells did not show significant differences ($p>0.05$ in One-way ANOVA; n=3 experiments). (B) Infection with RL1+/27+/4-pR19Nurr1 did not induce TH expression in cortical and striatal progenitor cells. 40x magnification.

5.3.4.3 Nurr1 induces TH expression in neurospheres generated from different regions of the CNS

As discussed previously, neurospheres generated from cortical and striatal precursor cells do not generate TH positive cells and only an insignificant number of dopaminergic neurons can be maintained from mesencephalic neurosphere cultures expanded in FGF2. Therefore, neurospheres represent an ideal system to perform gain-of-function studies. In order to establish if Nurr1 can induce TH expression in the presence of glial cell types the effects of virally delivered Nurr1 in neurospheres was examined.

Neurospheres generated from E14 cortex, striatum and ventral mesencephalon were expanded in FGF2 prior to transduction with RL1+/27+/4-pR19Nurr1wpre or controls. As proliferation of neural progenitor cells has been demonstrated to be important for the generation of TH expressing cells (Wagner et al., 1999), Nurr1 infected neurospheres were maintained in growth factor containing media for one day post transduction. Thereafter neurospheres were induced to differentiate by withdrawal of FGF2 for five days. Cells were characterized for TH expression by immunocytochemistry or western blot analysis performed as shown in Figure 5-15.

No TH expression was detected by immunocytochemistry or immunoblotting in uninfected neurospheres or after infection with control virus RL1+/27+/4-pR19GFPwpre. However, a significant number of TH expressing cells was detected in cortical and striatal neurospheres infected with Nurr1 expressing virus. As mesencephalic neurospheres can contain TH expressing dopaminergic neurons an increase of induction was difficult to assess by immunocytochemical staining. Since enzyme treatment and trituration may cause cell death and hence, loss of TH expressing cells, it was decided not to dissociate the neurospheres into single cells for quantification. Instead western blot analysis was performed confirming an upregulation of TH expression in cortical, striatal and mesencephalic neurospheres when infected with the Nurr1 expressing virus.

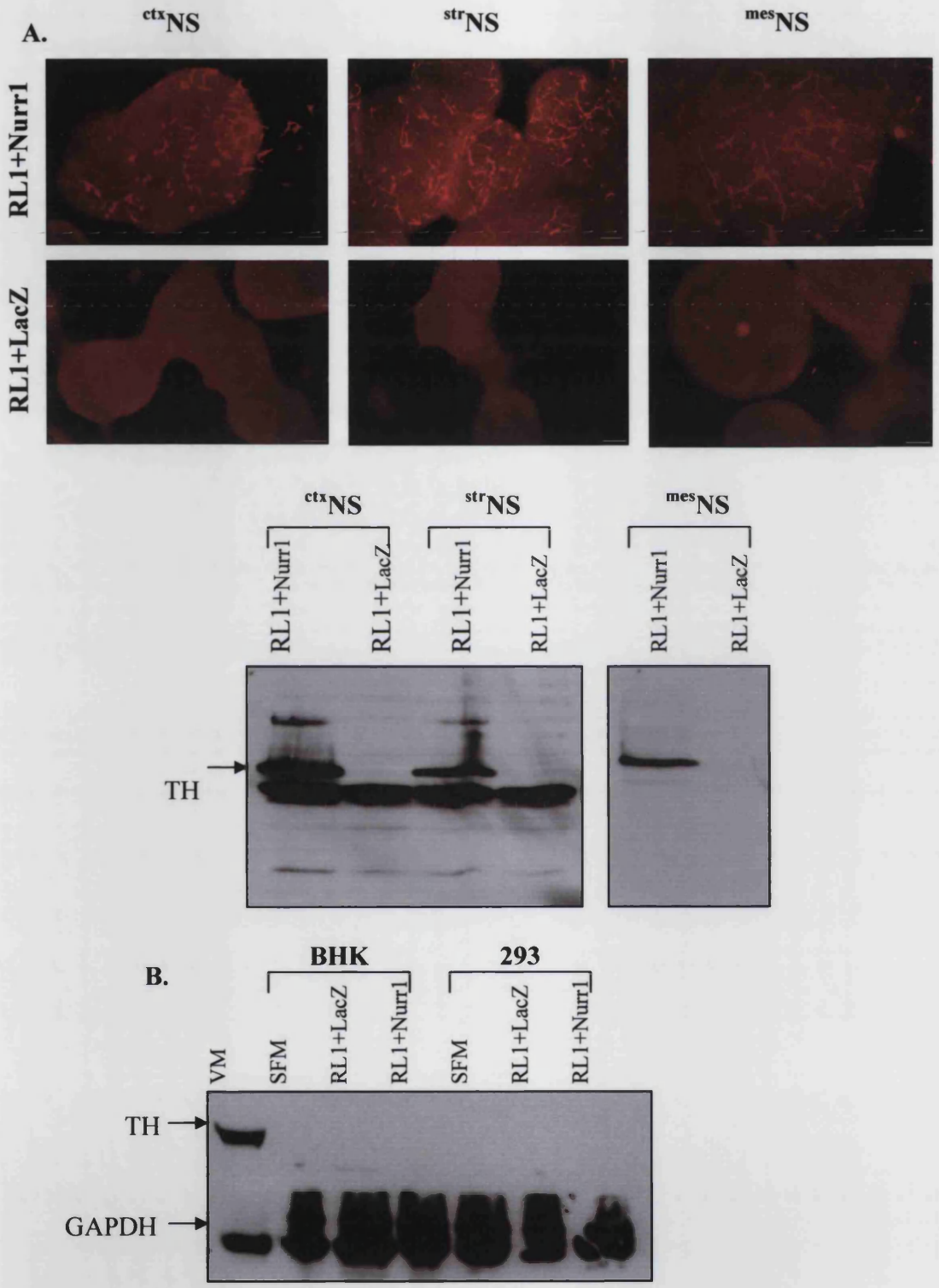


Figure 5-15: Virally delivered Nurr1 induces TH expression in neurospheres. (A) E14 derived and in FGF2 expanded neurospheres from cortex, striatum and ventral mesencephalon were infected with RL1+/27+/4-pR19Nurr1 or RL1+/27+/4-pR19LacZ. One day post infection growth factors were withdrawn and neurospheres were plated on PLL/Laminin coated coverslips and differentiated for five days. Cells were stained for TH (Alexa546, red). Neurospheres infected with Nurr1 expressing virus induced TH expression in neurospheres of all three neurogenic regions while controls did not contain TH expressing cells. Quantification by western blotting confirmed Nurr1 mediated TH induction in neurospheres. Scale bar represents 50µm. (B) TH induction is cell-context dependent as Nurr1 infection did not induce TH expression in BHK or 293 cells.

Since the data had not yet confirmed if TH upregulation is cell type dependent or if Nurr1 unspecifically upregulates TH expression independent from the cell context, BHK cells and 293 cells were infected with Nurr1 expressing virus or control virus. Western blotting for TH (Figure 5-15B) did not reveal an induction of TH expression in these cell types.

This gain-of-function study has demonstrated that virally delivered Nurr1 can significantly induce TH expression in a culture system containing a mixture of neural precursor cells, including neurons, astrocytes and oligodendrocytes. The induction of TH seems to be restricted to the tested neural precursor cells and hence, likely depends on the cell-context. Since ectopic TH expression does not necessarily imply that *bona fide* dopaminergic neurons have been generated, the nature of the TH expressing cell was characterized next.

5.3.4.4 Characterization of TH expressing cells

It was observed that Nurr1 mediated TH upregulation depended on the presence of proliferating conditions during infection as transferring infected neurospheres immediately into growth factor free culture conditions produced a reduced number TH expressing cells. This has also been described by others showing that infection of differentiated precursor cells with Nurr1 expressing constructs did not have any inductive effects (Kim et al., 2003a). To study if TH expressing cells have been generated *de novo* or if TH is upregulated in post-mitotic neural cell types, Nurr1 infected cortical neurospheres were BrdU pulse labelled during the first 24 hours after infection.

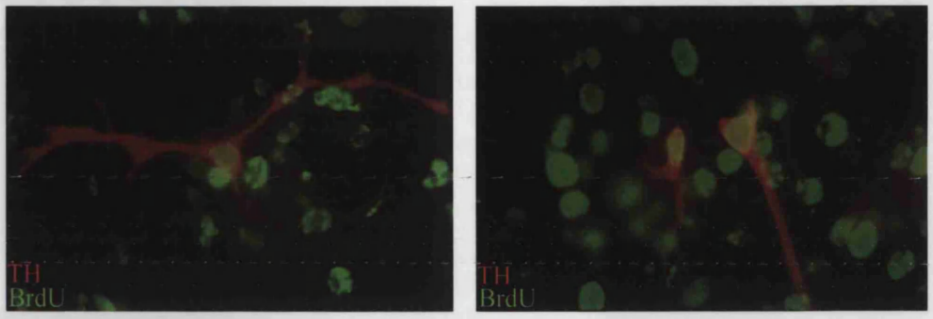
Immunocytochemical staining (Figure 5-16) confirmed that most of the TH expressing cells had incorporated BrdU and have therefore been generated from a proliferating precursor cell population. Differentiation into post-mitotic cell types appeared to occur after induction of TH expression in the cells. However, TH induction was not completely abolished when cell proliferation was inhibited in the presence of Ara-C (20μM) from the day of Nurr1 infection (data not shown). This

suggests that proliferation conditions during the infection phase are beneficial but not essential for Nurr1-mediated TH induction.

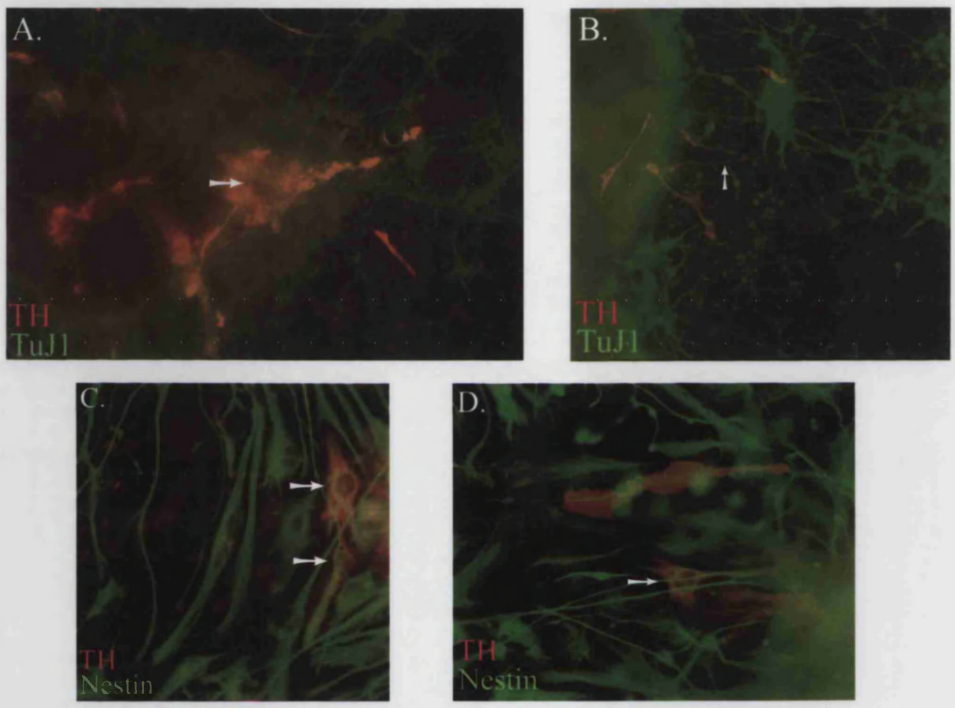
Nurr1-induced TH positive cells exhibited two distinct morphologies: (i) TH positive cells often arranged in clusters of mainly rounded cells with short processes (Figure 5-16) and (ii) as single TH positive cells with large cell body bearing usually 2-3 processes. However, the cell morphology of TH expressing cells did not resemble those of dopaminergic neurons generated from primary progenitor cultures. Indeed it was found by immunocytochemical staining that most of the TH expressing cells did not co-label with the neuronal marker TuJ1. Instead the majority of the TH expressing cells double labelled with nestin, some strongly while others revealed only a weak immunoreactivity for the neurofilament marker (Figure 5-16). Thus, the TH expressing cells may still be immature and longer differentiation periods may be necessary to acquire a fully mature neuronal cell fate. Antibody incompatibility did not allow double labelling for TH and GFAP.

Other studies have shown that ascorbic acid during culture period after Nurr1 transduction can significantly increase the number of TH positive cells (Kim et al., 2003a). TH expression of Nurr1 infected cortical neurospheres that were differentiated in the presence or absence of ascorbic acid (200μM) was quantified by western blot analysis (Figure 5-16). TH expression was significantly upregulated in the presence of ascorbic acid. These data suggest that AA acts in additive fashion on the Nurr1-induced generation of TH positive cells.

I. BrdU/TH double labelling of Nurr1-induced ^{ctx}NS



II. Morphology of Nurr1-induced TH expressing cells



III. Effects of ascorbic acid

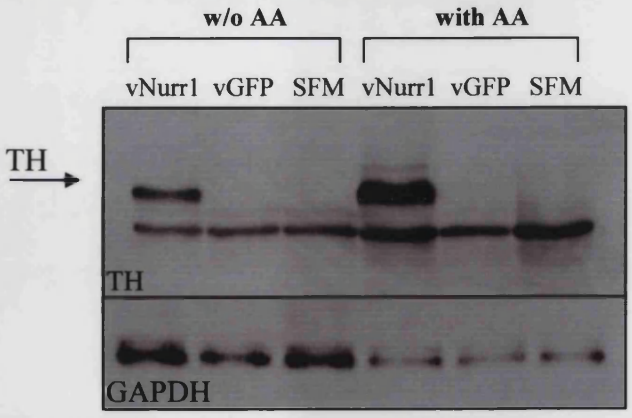


Figure 5-16: Characterization of Nurr1-induced TH expressing cells. (I) BrdU pulse labelling (0.2µM for 12h) revealed a high number of *de novo* generated TH expressing cells. (II) Immunocytochemical staining of Nurr1 infected ^{ctx}NS showed TH expressing cells (Alexa 546, red) often occurring in clusters (A, arrow). However, only a few of the TH expressing cells co-label with the neuronal marker TuJ1 (Alexa 488, green) (B, arrow). Most of the cells show low reactivity for nestin (C, D arrow). (III) Incubation with AA of ^{ctx}NS infected with RL1+/Nurr1 further increases TH expression as shown by western blot analysis.

5.3.5 Unaltered behavioural impairment of virally delivered ShhN and FGF2 in 6-OHDA lesioned animals

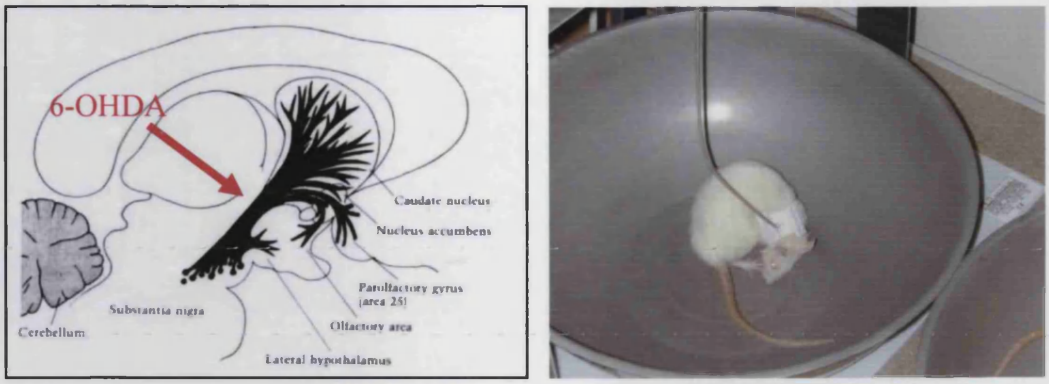
Effects of recombinant ShhN protein on behaviour improvement has previously been tested in 6-OHDA (Tsuboi and Shults, 2002) and MPP+ (Dass et al., 2002) animal models of Parkinson's disease. Tsuboi and Shults showed that repeated striatal administration of ShhN prior to injection of 6-OHDA into the striatum could reduce apomorphine and amphetamine induced rotations comparable to the effects of GDNF used as positive control in that study (Tsuboi and Shults, 2002). Surprisingly, only low concentrations of recombinant ShhN had a beneficial effect on behavioural impairment while high concentrations did not significantly decrease drug-induced rotations. Behavioural improvement was accompanied by an increased density of TH fibers in the respective groups but did not preserve the loss of dopaminergic neurons in the substantia nigra. As mentioned in the previous chapter, Shh appears to play a continuous role in the stem cell niche of the adult SVZ. While removal of Shh signalling significantly reduced the number of neural progenitors in the postnatal SVZ (Machold et al., 2003), striatal administration of ShhN protein increased the transcription levels of its receptor, patched, in the SVZ. However, this was without altering the proliferation or differentiation of SVZ precursor cells (Charytoniuk et al., 2002). These studies support the idea that the Shh signalling pathway may be activated *in vivo* in the adult rodent SVZ.

In order to establish if ShhN and FGF2 can improve behavioural impairment in 6-OHDA lesioned animals the effects of virally delivered factors was established. By delivering the FGF2 expressing virus it was intended to increase proliferation of endogenous precursor cells residing in the SVZ as has been demonstrated e.g. by intrastriatal administration of TGF-α (Fallon et al., 2000). With respect to a potential role of ShhN on the adult neural stem cell niche, it was anticipated that a combination of proliferating signals (FGF2) and inductive/differentiation clues (ShhN) may reduce behavioural impairment in this animal model of PD.

A severe depletion of dopaminergic neurons was induced unilaterally by injection of free base 6-OHDA (12.5μg) into the medial forebrain bundle. This acute chemical lesion resulted in a profound loss of dopaminergic neuron cell bodies in the substantia nigra and of their axons projecting into the striatum (Figure 5-17B). The loss of dopaminergic neurons was measured by apomorphine induced rotation behaviour using an automatic Rotorat™ system (Med Associates Inc.) to determine the number of fully completed contralateral rotations assessed over a 30min period after drug administration (0.5mg apomorphine per kg, s.c.). Animals rotating with more than 5 turns/min have more than 80% of the dopaminergic neurons lost (Ungerstedt, 1971b) and were selected for the experiment. As the rotational impairment varied over the first weeks after the 6-OHDA injection, rotational behaviour was determined every two weeks over a period of six weeks until animals showed stable rotational behaviour. Animals were grouped such that each group contained the same number of animals with increased rotations (more than 8 rotations/min) and animals with less profound degeneration (between 5 and 8 rotations/min). Each group (n=8 animals) performed the same number of total rotations/min prior to viral injection. $5x10^5$PFU of viral vectors were injected into the striatum ipsilateral to the 6-OHDA lesion. Groups were injected either with FGF2 expressing vector (1764/27-/4-/pR19FGF2), the ShhN expressing construct (1764/27-/4-pR19ShhN), a mixture of FGF2 and ShhN expressing vectors or with control virus expressing LacZ (1764/27-/4-pR19LacZ). Apomorphine induced rotational behaviour was determined every two weeks over a total period of 12 weeks (Figure 5-17).

None of the groups showed a significant improvement ($p>0.05$ compared to 1764/27-/4-pR19LacZ) in behavioral impairment over the tested period. Although administration with ShhN expressing vectors decreased the number of rotations within the first two weeks after virus injection, the trend was not maintained but actually reversed twelve weeks after inoculation. Analysis of the rotational behaviour of single animals rather than the entire group also did not reveal any potential beneficial effects of the administrated viral vectors.

A.



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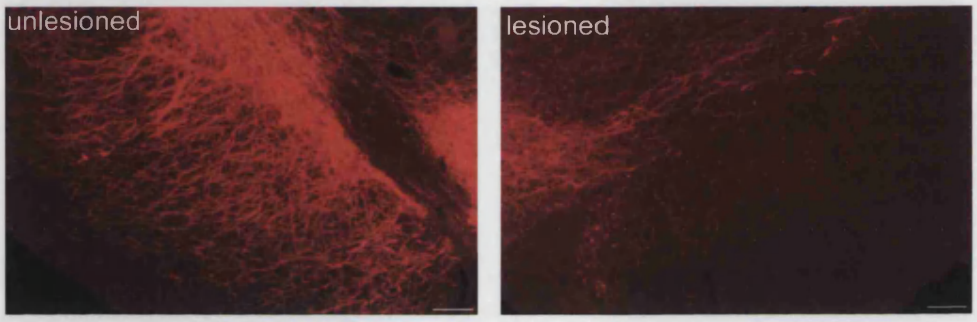


C.

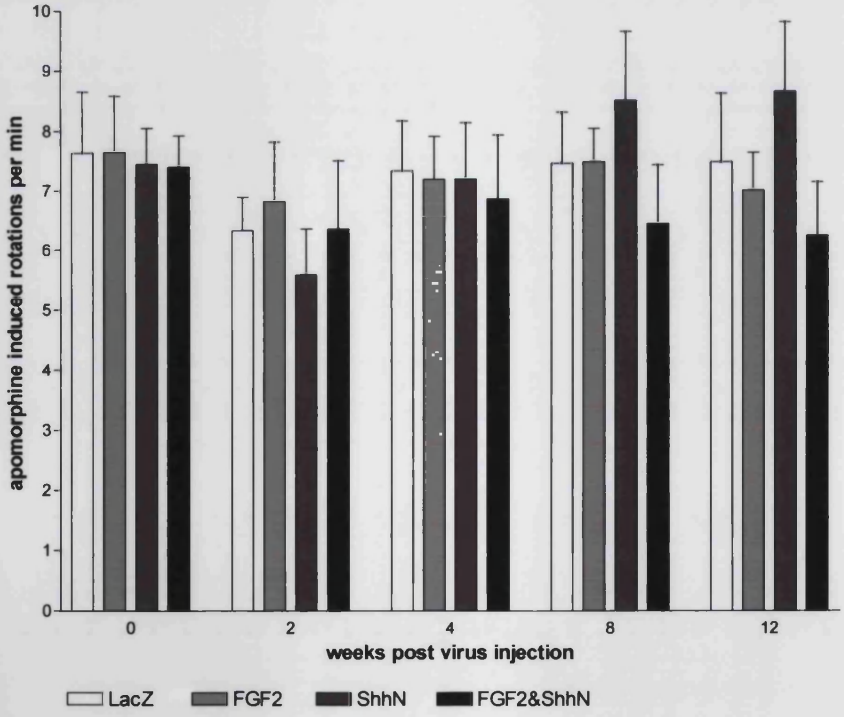
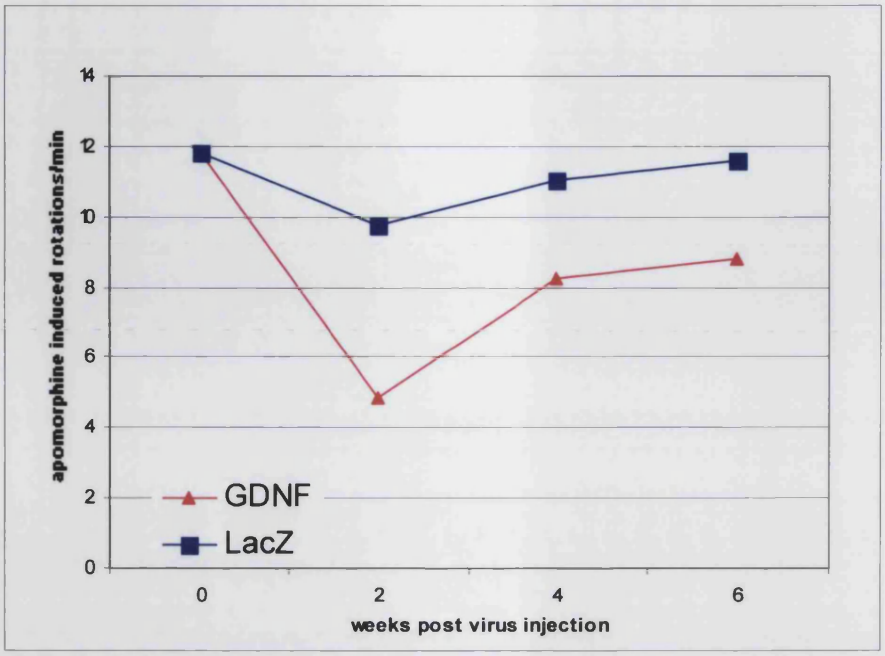


Figure 5-17: Effects of virally delivered FGF2 and ShhN on rotational behaviour after 6-OHDA lesion. (A) In the animal model of PD an acute lesion of the nigrostriatal pathway was induced by injection of 6-OHDA into the medial forebrain bundle. Apomorphine induced rotational behaviour was monitored with an automatic Rotorat® system. (B) Immunohistochemical staining for TH shows depletion of dopaminergic neurons in the SN. (C) Apomorphine induced rotational behaviour of 6-OHDA lesioned animals after intrastriatal injections of highly disabled 1764/27-/4- expressing FGF2, ShhN, FGF2 and ShhN or LacZ. No improvement of rotational impairment was observed.

The experimental rationale was tested by injecting viral vectors expressing glial derived neurotrophic factor (GDNF) (1764/27-/4-pR19GDNF) into the striatum of 6-OHDA lesioned animals four weeks after depletion of the nigrostriatal pathway (Figure 5-18). GDNF has been well established to improve behavioural impairment in this animal model by administration either prior or post 6-OHDA lesion (reviewed by Bjorklund et al., 2000). Indeed a significant improvement in apomorphine induced contralateral rotational behaviour was observed with the GDNF expressing vector two weeks after virus injection ($p<0.05$ vs. LacZ expressing vector in two-paired t test; n=6 animals). However, the improvement decreased over time and was less prominent 6 weeks post virus administration.

Intrastriatal delivery of FGF2 expressing virus was also tested for any ability to increase cell proliferation. Unlesioned animals were either injected with 1764/27-/4-pR19FGF2 or 1764/27-/4-pR19LacZ (n=4 animals per treatment) and BrdU (100mg/kg; i.p.) over a period of six consecutive days. Immunohistochemical staining revealed an increased number of BrdU positive cells in the striatum of FGF2 injected animals, but no profound increase in control animals. The striatum of the FGF2 group also contained a large number of nestin positive cells. Interestingly, a significantly increased number of BrdU positive and nestin positive cells were also found in the substantia nigra of FGF2 injected animals. BrdU positive cells were only observed ipsilateral to the injection site and not in the control group injected with LacZ expressing vector. It has previously been shown that the less disabled vector used in this experiment efficiently expresses transgenes in the substantia nigra after intrastriatal injections (Lilley et al., 2001). Retrograde transport of the virus into the cell body of dopaminergic neurons after infection of striatal terminals make this vector particularly amenable for gene delivery to the substantia nigra. Hence, overexpression of FGF2 in the substantia nigra may have induced cell proliferation in this region. However, immunohistochemical doublestaining for BrdU and GFAP, nestin or TuJ1 failed to identify the antigenic character of the generated cells.

A.



B.

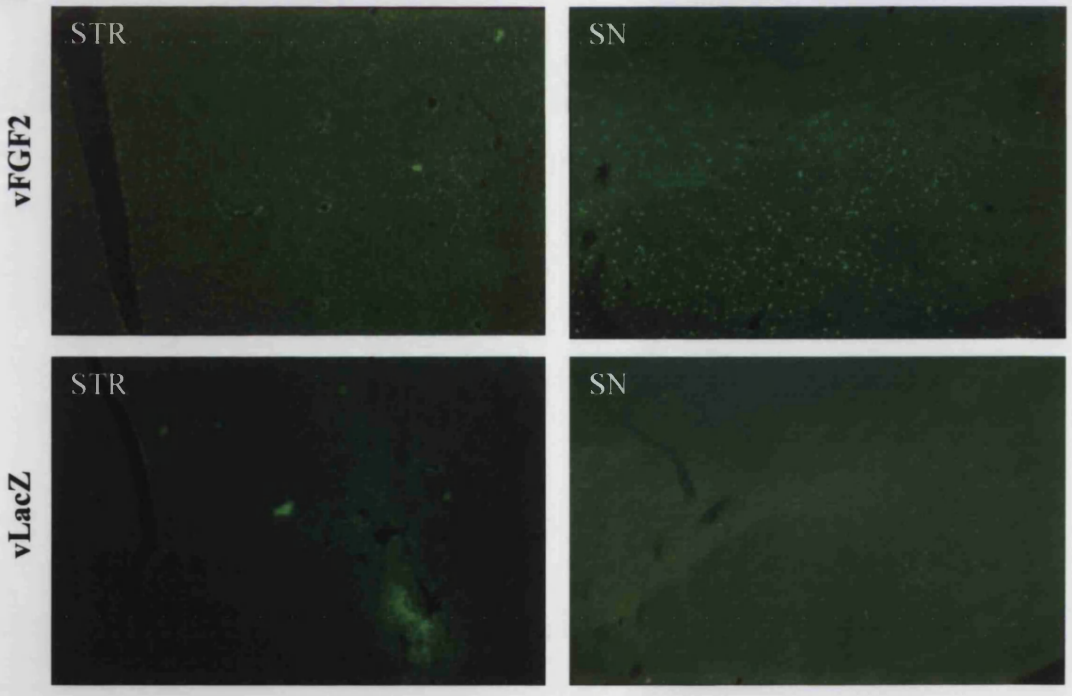


Figure 5-18: Intrastriatal injections of GDNF and bFGF. (A) Intrastriatal injections of GDNF expressing virus 1764/27-/4-/pR19GDNF after 6-OHDA lesion of the MFB reduced apomorphine induced rotational behaviour. (B) Intrastriatal injections of 1764/27-/4-/pR19FGF2 increased cell proliferation in the striatum and substantia nigra in unlesioned animals as shown by immunhistochemistry for BrdU (green). STR=striatum, SN=substantia nigra.

5.4 Discussion

In an attempt to examine the roles of selected factors on the induction of TH expressing dopaminergic phenotypes from neural precursor cells, disabled HSV-1 were constructed delivering FGF2, FGF8, ShhN and Nurr1, respectively. Under defined, serum-free conditions their function was studied in two *in vitro* cell models, glial deprived neural progenitor cultures or glial containing neurosphere cultures. Selected vectors were tested in an animal model of Parkinson's disease.

Virally expressed FGF2 and FGF8 maintain progenitor cells in a proliferative state

Virally delivered FGF2 and FGF8 were expressed, posttranslationally modified and bioactive as demonstrated by inducing differentiation of PC12 cells. In neural progenitor cells the virally delivered factors induced proliferation of the otherwise post-mitotic neuronal cell population and FGF8 additionally exerted survival effects on primary midbrain dopaminergic neurons. The neurotrophic effects of FGF8 on primary mesencephalic progenitor cultures further confirmed data presented in the previous chapter suggesting selective effects on dopamine neurons generated from neurospheres. However, virally delivered FGF2 did not increase the number of dopaminergic neurons as previously demonstrated by others suggesting increased proliferation of E12 dopamine neuroblasts (Bouvier and Mytilineou, 1995) and survival of E15 primary dopaminergic neurons (Engele, 1998a). Daadi and Weiss (1999) even suggested inductive effects on *de novo* generation of dopaminergic neurons from E14 cortical and striatal progenitor cells mediated by FGF2. However, with 0.31% of the total striatal cell population expressing TH this appears to be a rather rare event and significant TH expression in cortical and striatal progenitor cells could only be induced by synergistic effects of FGF with media conditioned by astrocytes. Further the synergistic effects of FGF2 and the conditioned media (CM) depended on the time point of availability as supplementation 24 hours after plating did not exert significant TH induction (Daadi and Weiss, 1999). TH induction was not found in any of the forebrain cultures following delivery of FGF2 or FGF8. Since the chosen serum-free culture conditions did not support proliferation or differentiation of

astrocytes, the inductive factors released by glia cells may simply not be present in the culture conditions. Indeed it was shown by immuncytochemistry that virally delivered FGF2 and FGF8 maintain a dividing population of undifferentiated, nestin positive cells but had no effects on gliogenesis. As the promoter constructs used in this study did not allow regulated gene expression, and transgene expression continued for extended periods *in vitro*, the infected cultures were sustained in this mitogenic state. Supplementation with glial conditioned media would be interesting, but was not carried out in the study.

Glial released factors may facilitate the TH induction by Nurr1

The molecular character of a potentially inductive glial derived factor(s) still remains unidentified. While a crucial role of these factors for the induction of a dopaminergic phenotype is undisputable, selective (survival) and/or instructive (differentiation) functions have been suggested: (i) BrdU/TH double labelling favored the idea of *de novo* generated dopaminergic neurons induced by the presence of CM in forebrain progenitor cultures (Daadi and Weiss, 1999) and Nurr1-expressing neural stem cell lines (Wagner et al., 1999). Testing different mitogens and morphogens the inductive effects of CM could not be mimicked in the mentioned studies. (ii) Survival effects on midbrain dopaminergic neurons were facilitated by CM from forebrain and midbrain glial cultures (Engele and Bohn, 1991; Engele, 1998b), independent from the embryonic stage from which the CM was generated (Pei et al., 2003), and were also achieved by co-cultures of mesencephalic cultures with striatal O-2A progenitor cells (Takeshima et al., 1994a) or type-1 astrocytes (Takeshima et al., 1994b).

Factors released from glial cells may also explain the observed different inductive effects on TH expression of Nurr1 delivering vectors. Infection of forebrain and midbrain progenitor cells that have proven to lack a significant number of astrocytes did not induce TH, while neurosphere cultures from the same neurogenic regions demonstrated ectopic TH expression after delivery of Nurr1. These finding are in agreement with other reports demonstrating that Nurr1 failed to induce TH expression in cortical progenitor cells that had been differentiated for four days prior to transfection (Kim et al., 2003a). However, the authors argue that this is due to the

post-mitotic state of the cultures and that DA-inducing effect of Nurr1 requires precursor proliferation.

With respect to work presented here, it was found that CNS progenitors were mitotic active at least within the first three days, and BrdU labelling revealed that about 10% (mesencephalic) to 30% (cortical) of the progenitor cells were proliferating despite the lack of exogenous growth factors. In a preliminary experiment, inhibition of cell division by addition of Ara-C did not abolish the Nurr1 mediated inductive effects in cortical and striatal neurosphere cultures. Therefore, it does not appear that the lack of proliferative conditions is the main reason for the failure of TH induction but that factors not present in progenitor cultures but released in neurosphere cultures explain this discrepancy. Interestingly, most of the TH expressing cells did not co-label with the neuronal marker TuJ1. Other groups have proposed that Nurr1 solely enhances the expression of TH without accompanying neural differentiation (Sakurada et al., 1999) by direct activation of the TH gene promoter (Iwawaki et al., 2000). Kim *et al.* (2003) also found that only a minority of Nurr1-induced TH positive cells acquire neuronal characteristics (12% co-label with MAP2 and 23% with TuJ1). Longer or enforced differentiation did not affect the number of TH positive cells expressing neuronal markers. However, co-localization with GFAP was also not detected (Kim et al., 2003a). It is also intriguing that the cell morphology of TH expressing cortical and striatal progenitor cells is very distinctive as compared to that observed in primary derived mesencephalic dopaminergic neurons. Nurr1-induced TH expressing cells appeared to have less and shorter processes with spinal shaped cell bodies. Overall it appears that the TH expressing cells did not have the characteristics of *bona fide* dopaminergic neurons or may alternatively be immature dopaminergic neurons as proposed by others (Kim et al., 2003a). In fact, some of the Nurr1-transduced TH positive cells were demonstrated to incorporate BrdU and to be nestin positive suggesting precursor status.

Virally delivered ShhN is not sufficient to induce dopaminergic phenotypes

The inductive role of Shh on the development of midbrain dopaminergic neurons *in vivo* (Wang et al., 1995; Hynes et al., 1995a) is undisputable and has prompted many scientists to examine potential implication on TH expression in neural progenitor and

neural stem cells *in vitro*. In primary mesencephalic cultures Shh has been suggested to be neurotrophic for the survival of dopaminergic neurons as high concentrations of the protein increased the number of TH expressing cells, confirmed by protective effects in MPP+ cytotoxicity studies and blocking experiments with Shh antibodies (Miao et al., 1997). BrdU incorporation assays did not support the idea that Shh induces a dopaminergic phenotype in mesencephalic progenitor cells (Miao et al., 1997), as it has also been shown for forebrain (Daadi and Weiss, 1999; Stull and Iacovitti, 2001) and hippocampal (Sakurada et al., 1999) progenitor cells.

In this chapter it has been shown that virally delivered ShhN did not induce TH expression in cortical or striatal progenitor cells. However, neither did it exert neurotrophic effects on the survival of midbrain dopaminergic progenitors. Although expression and bioactivity of virally delivered ShhN have been validated, this result is not in agreement with other studies (Miao et al., 1997) and may be explained by insufficient expression levels as discussed. With respect to findings presented in the previous chapter showing an increase in TH expression when ShhN is added during expansion phase of E12 mesencephalic precursor cells, the proliferative state and the presence of other (glial released) factors may determine the observed effect. It has also been stated by others that "Shh may play an important role in the early patterning of proliferative precursors but may have an inhibitory effect on TH expression when expressed during the terminal stages of differentiation" (Sakurada et al., 1999) and that "Shh exerts its effects *in vitro* on the still dividing b-FGF stimulated neural precursors" (Volpicelli et al., 2004). Synergistic effects of ShhN and FGF8b on TH induction have been reported *in vivo* (Ye et al., 1998) and also for an immortalised mouse neural stem cell line (Kim et al., 2003c), but were not able to induce dopaminergic phenotypes in mouse striatal progenitor cells and human neural stem cell lines (Stull and Iacovitti, 2001). However, the situation is very different in embryonic stem cells as compelling evidence has proven inductive effects mediated by these factors to generate functional dopaminergic neurons (Lee et al., 2000; Kim et al., 2002; Lin and Rosenthal, 2003).

Overall, this chapter has demonstrated that the cell system used allows determination of the inductive effects of selective factors, such as FGF2, FGF8, ShhN or Nurr1. Neural precursor cells generated from the same neurogenic region and embryonic age

showed very distinctive susceptibilities to such signals when maintained as primary neuronal and progenitor cultures compared to neurosphere cultures. This may result from the proliferative and differentiative state of the precursor cells, as well as from the release of factors conditioned by glial cells present or absent in the respective *in vitro* cell model.

Virally delivered FGF2 appeared to increase cell proliferation in vivo but neither FGF2 nor ShhN improved rotational impairment

The idea of stimulating endogenous stem cells of the SVZ to proliferate and differentiate *in situ* into neuronal cell types, thereby replacing lost or damaged dopaminergic neurons, is possibly the most challenging experimental approach to Parkinson's disease therapy. On the other hand, limited scientific experience in studying functional replacement by using endogenous stem cells make experiments highly speculative and novel. A limited number of reports have described possible approaches to study endogenous neurogenesis in the substantia nigra. Fallon *et al.* demonstrated that intrastriatal administration of TGFα, which activates the EGF receptor (EGFR) (Tropepe et al., 1997), induced a massive cell proliferation originating from the SVZ and leading to the mitogen source in 6-OHDA lesioned animals (Fallon et al., 2000). Signalling via EGF-R induces rapid proliferation of transit-amplifying C cells (Doetsch et al., 2002) and therefore the observed cell proliferation is likely to be attributable to increased amplification of type C cells. Adding to previous work on *in vivo* proliferative effects of TGFα (Craig et al., 1996; Tropepe et al., 1997), Fallon *et al.* showed that combination of an acute insult (6-OHDA lesion) and growth factor administration can guide precursor cells to the site of injury, and most notably is accompanied by the appearance of TH positive cells in the striatum and improvement of behaviour impairment. However, Fallon *et al.* did not quantify the number of generated TH positive neurons appearing in this ectopic position (striatum), and behavioural improvement is most likely not due to reconstitution of the nigrostriatal pathway. Zhao *et al.* suggested the evidence of dopamine neurogenesis in the substantia nigra as a rare event that can be increased after injury based on confocal analysis of TH/BrdU double labelled cells (Zhao et al., 2003). Using DiI-labelling Zhao *et al.* proposed that the cells giving rise to

dopaminergic neurons in the substantia nigra originate from the ventricular system in the midbrain and upon differentiation by unknown endogenous inducers, integrate into the basal ganglia circuitry. However, using a similar experimental approach as Zhao *et al.* the generation of new dopaminergic neurons in the adult substantia nigra was not evident in other studies (Frielingsdorf et al., 2004).

In work performed prior to publication of these reports the potential effects of virally delivered ShhN and FGF2 on behavioural recovery in a rat model of Parkinson's disease was examined. To avoid neuroprotective effects of ShhN (Miao et al., 1997) transgene expressing vectors (FGF2 and ShhN) were injected after depletion of the nigrostriatal pathway with 6-OHDA. Further, gene delivery was performed six weeks after the toxic insult when apomorphine induced rotational behaviour was stable and depletion had become fully established. Medial forebrain bundle lesions with 6-OHDA resulted in an almost entire denervation compared to partial striatal lesions (Deumens et al., 2002). With respect to this complete unilateral destruction of the dopamine pathway no recovery effects were observed resulting from virally delivered ShhN and FGF2. The insult might have been too severe to generate enough dopamine releasing cells in order to achieve recovery. However, viral delivery of GDNF into 6-OHDA lesioned animals did show significant ($p<0.05$; n=6 animals two weeks after virus injection) improvement in rotational behaviour.

Interestingly, an increased number of BrdU positive cells was found in the striatum and substantia nigra of FGF2 injected animals. Although a ridge of migrating cells was not observed as Fallon *et al.* described by injecting TGFα, the anterior SVZ appeared thickened and contained more BrdU positive cells than contralateral to the injection site or in LacZ injected animals. Therefore the proliferated cells may have been derived from stem cells lining the ventricular system giving rise to possibly rapidly dividing type C cells migrating to other regions of the forebrain and midbrain. However, it has proven difficult to identify the antigenic identiy of the generated cells. Although an increased number of nestin positive cells was detected in the striatum and the substantia nigra, antibodies used at that time of the study did not allow convincing co-labelling with BrdU immunohistochemistry. The high number of BrdU labelled cells found in the substantia nigra after FGF2 delivery was surprising.

The cells may have originated from stem cells lining the ventricle system dorsal of the substantia nigra (Zhao et al., 2003) or residing within the substantia nigra (Lie et al., 2002). Co-labelling of BrdU and TH did not reveal any evidence of *de novo* generated dopaminergic neurons in this study which is in agreement with a recent report (Frielingsdorf et al., 2004). Interestingly, Lie *et al.* demonstrated that progenitor cells residing in the substantia nigra can give rise to all neural cell types when isolated and propagated *in vitro* but do not generate neurons *in vivo* (Lie et al., 2002). Controversially, Zhao *et al.* propose unknown endogenous signals inducing differentiation of precursors into TH expressing neurons while Lie *et al.* discussing repressive signals in the substantia nigra inhibiting neurogenesis in this region. Species differences (Zhao *et al.* were using mice while Lie *at al.* were using rats) have been suggested to explain this dilemma (Zhao et al., 2003) and could also be considered for the missing differentiation of proliferated cells in this rat study.

Overall, the experiments have shown that although cell proliferation in the substantia nigra can be induced by virally delivered FGF2, virally delivered ShhN alone or in combination with FGF2 appear insufficient to recover dopamine function in the animal model used. Considering recent findings that Shh does have an impact in the adult SVZ (Charytoniuk et al., 2002; Machold et al., 2003), and that neurogenesis might be possible in the denervated striatum and substantia nigra (Fallon et al., 2000; Zhao et al., 2003), it might be wise to combine Shh with other factors, such as Nurr1 or FGF8. From an experimental perspective it could be beneficial to enhance precursor proliferation prior to delivery of differentiative signals. As we are at the very beginning of the understanding of the mechanisms of endogenous stem cell behaviour, future findings will hopefully reveal the factors inducing or repressing neurogenesis in the adult substantia nigra.

FINAL CONCLUSION

The adult mammalian brain cannot regenerate. This is an old dogma that needs correcting in light of recent findings that neurogenesis is not restricted to the developing nervous system, but also occurs in the adult brain. The expansion and controlled conversion of neural stem cells into dopaminergic neurons has potential use for *in vivo* and *ex vivo* approaches in the treatment of Parkinson's disease. However, methods by which precursor cells might be differentiated into a dopaminergic phenotype are unknown. An understanding of the natural differentiation stimuli may help to develop systems for the controlled generation of dopaminergic neurons in high yields. With progress in the development of replication deficient viruses, vector systems have become available to deliver the genetic information required for the possible induction of a dopaminergic phenotype. For this purpose various viral vector systems have been used, but no reports have previously thoroughly studied the use of HSV-1. However, HSV-1 has advantages compared to other viral vectors as a potential gene delivery system for neural precursor cells as the lack of chromosomal integration avoids the possible disruption of the host genes and the establishment of a latency-like state upon infection allows long-term transgene expression without clearance of virally infected cells by the host immune system.

The work presented in this thesis aimed to investigate the potential use of HSV-1 based vectors for the delivery of transgenes to neural precursor cells of the CNS. Replication disabled HSV-1 vectors were used to deliver genes encoding factors that play important roles in the development of dopaminergic neurons. Their role for the induction of a dopaminergic phenotype from neural precursor cells was studied.

Chapter 3 demonstrated that disabled HSV-1 vectors can be used to deliver genes of interest to neural precursor cells. However, efficiency of gene delivery depended on the disablement of the vector and the cell system used. Highly disabled vectors resulted in insufficient transgene expression in primary neuronal and neural progenitor cultures, but efficient reporter gene expression in neural precursor cells expanded as neurosphere cultures. The integrity of the neural precursor cells, e.g. multipotency and self-renewal capacity, were not affected by viral transduction. Less disabled vectors expressing ICP27 and ICP34.5 gave stronger reporter gene expression in primary neural and neurosphere cultures, but on the expense of an

increased neuronal cell loss in these models most likely due to expression of toxic viral gene products. Finally it was shown that replication-deficient HSV-1 also delivered transgenes to cells within the stem cell niche *in vivo*. Thus, future applications to alter the behaviour of these cells *in situ* may become possible.

Chapter 4 revealed that FGF8 is sufficient as mitogen for the expansion of neural precursor cells *in vitro*. Most notably FGF8 increased the number of dopamine neuroblasts and/or existing primary dopaminergic neurons. This was, however, most likely not due to inductive or proliferative effects, but due to enhanced survival. Interestingly, results from this and the following chapter indicated that FGF8 expanded or infected neurospheres tended to generate an increased number of oligodendrocytes. Although this phenomenon has not further been investigated, it would be interesting to consider in future studies.

In chapter 5 replication-deficient HSV-1 vectors were constructed expressing factors known to be involved in dopamine neurogenesis from embryonic precursor cells during development *in vivo* and from embryonic stem cells *in vitro*. Summarising the results of this and the previous chapter regarding dopamine neurogenesis, it was found that none of the factors induced the *de novo* generation of dopaminergic neurons from neural precursor cells. Although FGF8 had survival effects on dopamine neurons and neuroblasts, and Nurr1 induced ectopic TH expression in precursor cells, the tested factors were not sufficient to generate new neurons with a midbrain dopaminergic phenotype. Interestingly, the inductive effects of Nurr1 on TH expression appeared to require the presence of glial cells or molecules released from these cells. Obviously, therefore, other factors are required to act synergistically with Nurr1 or the morphogens Shh and FGF8 on the conversion of a multipotent precursor cell into a dopaminergic neuron., but these remain to be identified.

A general limitiation encountered with the use of mesencephalic derived neurospheres is the low number of neurons generated from this region compared to striatal or cortical precursor cells. As shown in this study as well as by others (Ostenfeld et al., 2002a), continuously expanded mesencephalic neurospheres mainly produce glial cell types upon differentiation. The lack of pro-neuronal signals may partly be responsible

for the limited generation of dopaminergic neurons from this region. Future approaches could involve the delivery of pro-neuronal genes, such as neurogenin (Ma et al., 1996; Sun et al., 2001), to direct the fate of mesencephalic precursor cells into a neuronal phenotype prior to differentiation into dopamine neurons. However, since this approach would require a stable expression of the transcription factor over multiple passages of the precursor cells, integrative viral vectors may be more suitable than HSV-1.

The results generated lead to a fundamental concern. How similar is neurogenesis occuring in embryos to that occurring in *in vitro* cell models or in the adult CNS? Precursor cells maintained in culture, and the cell source of new neurons in the adult CNS, are unlikely to be direct counterparts of the neuroepithelial cells that generate dopaminergic neurons in the embryo. However, cell types retained in the neuroepithelium of the adult nervous system have similarities to embryonic neuroepethelial cells from which they are derived (reviewed in Alvarez-Buylla et al., 2001). It is even more intriguing that morphogens, such as Shh, Noggin, and BMPs, that are so critical to neurogenesis in the embryo, also appear to play a role in the adult neurogenic stem cell niches (reviewed in Alvarez-Buylla and Lim, 2004). It will be the challenge of future work to unveil the mechanisms to activate this cascade of morphogenic factors that may be involved in the *de novo* generation of neurons in the adult CNS.

The results presented in this thesis have identified disabled HSV-1 capable of gene delivery to neural precursor cells *in vitro* and in *vivo*. Various factors were tested for their potential to convert neural precursor cells into a dopaminergic phenotype, but only virally delivered Nurr1 induced the expression of the dopaminergic marker tyrosine hydroxylase. Future experiments may reveal if other factors are sufficient for the *de novo* generation of dopaminergic neurons from foetal neural precursor cells.

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APPENDIX

A.1 Sequence of basic fibroblast growth factor (FGF2)

Sequence file name:	A031000_C05_H2SP6_035.ab1.1
Plasmid:	pGemTbFGF
Squencing primer:	SP6
According Genebank accession number:	M22427

TTTTNAGNGACCCATCCAACGCGTTGGGAGCTCTCCCATATGGTCGACCTG
CAGGCGGCCGCGAATTCACTAGTGATTCGTAAAGCTTGATTCCATGGCTG
CCGGCAGCATCACTTCGCTTCCCGCACTGCCGGAGGACGGCGGCGGCGCC
TTCCCACCCGGCCACTTCAAGGATCCCAAGCGGCTCTACTGCAAGAACGG
CGGCTTCTTCCTGCGCATCCATCCAGACGGCCGCGTGGACGGCGTCCGGG
AGAAGAGCGACCCACACGTCAAACTACAGCTCCAAGCAGAAGAGAGAGG
AGTTGTGTCCATCAAGGGAGTGTGTGCGAACCGGTACCTGGCTATGAAGG
AAGATGGACGGCTGCTGGCTTCTAAGTGTGTTACAGAAGAGTGTTTCTTCT
TTGAACGCCTGGAGTCCAATAACTACAACACTTACCGGTCACGGAAATAC
TCCAGTTGGTATGTGGCACTGAAACGAACTGGGCAGTATAAACTCGGATC
CAAAACGGGGCCTGGACAGAAGGCCATACTGTTTCTTCCAATGTCTGCTA
AGAGCTGACTCTCTTTAGACACTGTCACTCTCAGGGAATCTCGAGTACGA
ATCGAATTCCCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCC
CAATTCGCCCTATAGTGAGTCGTATTACAATTNACTGGCCGTNGTTTTACA
ACGTCNGTGACTGGGAAAACCCCTGGCGTTCCCAACTTAAATCNNCTTGC
ANCACATNCCCCTTTCGCCAGNTGGCGT

Appendix 1: Sequence of rat basic fribroblast growth factor (bFGF or FGF2) for SP6 primers. In red idicating the start codon, in blue the stop codon. Underlined is part of the primer sequence used for the PCR reaction. The sequence was also confirmed using reverse sequence with T7 primers but the result was not illustrated here.

A.2 Sequence of rat aminoterminal sonic hedgehog (ShhN)

Sequence file name:	B170300_G06_BH6SP6_051.ab1
Plasmid:	pGemTShhN
Squencing primer:	SP6
According Genebank accession number:	L27340 and NM_017221

NNNTNGGGAACNCNCCCCCCGCTNTTGGGGGGGAACNCCNCCNNCCGNC
CNGCAGGCGGCCGCGAATNCTTTTGTGGGGCGGGGNAANCCCATTCCGCA
CCAGCTCGCGCACAGACCGGCGCGGGGACGGCTCGCAAGTCCTCAGGTTC
CGCGGACGAGATGCTGCTGCTGCTGGCCAGATGTTTTCTGGTGGCCCTTGC
TTCCTCGCTGCTGGTGTGCCCCGGACTGGCCTGTGGGCCCGGCAGGGGGTT
TGGAAAGAGGCGGCACCCCAAAAAGCTGACCCCTTTAGCCGTAAAGCAGT
TTATCCCCAACGTAGCCGAGAAGACCCTAGGGGCCAGCGGCCGATATGAA
GGGAAGATCACAAGAAACTCCGAACGATTTAAGGAACTCACCCCCAATTA
CAACCCCGACATCATATTTAAGGATGAGGAAAACACTGGAGCAGACCGGC
TGATGACTCAGAGGTGCAAAGACAAGTTAAATGCCTTGGCCATCTCCGTG
ATGAACCAGTGGCCTGGAGTGAAGCTTCGAGTGACTGAGGGCTGGGATGA
GGACGCCATCATTCAGAGGAGTCTCTACACTATGAGGGTCGAGCAGTGGA
CATCACCACGTCTGACAGGGACCGCAGCAAGTATGGCATGCTGGCTCGCC
TGGCTGTGGAGGCTGGCTTCGACTGGGTCTACTATGAATCCAAAGCTCAC
ATCCACTGCTCTGTGAAAGCAGAGAACTCCGTGGCGGCCAAATCTGACGG
CTGAAAAAAAAANAAAANA

Appendix 2: Sequence of rat aminoterminal sonic hedgehog (ShhN) for SP6 primers. In red idicating the start codon, in blue the stop codon. Turquoise codons indicate silent mutations and the green codon a polymorphism for rat ShhN in agreement with the sequence published in the Genebank accession number NM_017221. Underlined is part of the primer sequence used for the PCR reaction. The sequence was also confirmed using reverse sequence with T7 primers but the result was not illustrated here.

A.3 Sequence of rat Nurr1

Sequence file names:	A160500_G10_BH7P7_083.ab1.1
	A090600_E05_BH5P5_038.ab1.1
	A130400_E02_BH11_018.ab1
	B170300_B06_BH1T7_048.ab1
Plasmid:	pGemTNurr1
Squencing primer:	T7, SP6, gene specific as described in 2.2.11
According Genebank accession number:	U72345

CTCTNTTNGGGAACTTCGCCCCCCGCTTTTGGGGGGCAACNCCGGCCNCG
GGAATNNGNNTNAGCTTTCGGCTGTTNCCATGCCTTGTGTTCAGGCGCAGT
ATGGGTCCTCGCCTCAAGGAGCCAGCCCCGCTTCTCAGAGCTACAGTTAC
CACTCTTCGGGAGAATACAGCTCCGATTTCTTAACTCCAGAGTTTGTCAAG
TTTAGCATGGACCTCACCAACACTGAAATTACTGCCACCACTTCTCTCCCC
AGCTTCAGTACCTTTATGGACAACTACAGCACAGGCTACGACGTCAAGCC
ACCTTGCTTGTACCAAATGCCCCTGTCCGGACAGCAGTCCTCCATTAAGGT
AGAAGACATTCAGATGCACAACTACCAGCAACACAGCCACCTGCCCCCTC
AGTCCGAGGAGATGATGCCACACAGCGGGTCGGTTTACTACAAGCCCTCT
TCGCCCCCGACACCCAGCACCCCGGGCTTCCAGGTGCAGCATAGCCCGAT
GTGGGACGATCCGGGCTCCCTTCACAACTTCCACCAGAACTACGTGGCCA
CTACGCATATGATCGAGCAGAGGAAGACACCTGTCTCCCGCCTTTCACTCT
TCTCCTTTAAGCAGTCGCCCCCGGGCACTCCTGTGTCTAGCTGCCAGATGC
GCTTTGACGGGCCTCTGCACGTCCCCATGAACCCGGAGCCCGCGGGCAGC
CACCACGTAGTGGATGGGCAGACCTTCGCCGTGCCCAATCCCATTCGCAA
GCCGGCATCCATGGGCTTCCCGGGCCTGCAGATCGGCCACGCGTCGCAGT
TGCTTGACACGCAGGTGCCCTCGCCGCCGTCCCGGGGCTCTCCCTCCAATG
AGGGTCTGTGCGCTGTTTGCGGTGACAACGCGGCCTGTCAGCATTACGGT
GTTCGCACTTGTGAGGGCTGCAAAGGTTTCTTTAAGCGCACGGTGCAAAA
AAACGCGAAATATGTGTGTTTAGCAAATAAAAATTGCCCAGTGGATAAGC
GCCGCCGAAATCGTTGTCAGTACTGTCGGTTTCAGAAGTGCCTGGCTGTTG
GGATGGTTAAAGAAGTGGTTCGCACGGACAGTTTAAAAGGCCGGAGAGG

TCGTCTACCCTCAAAACCGAAGAGCCCACAGGATCCCTCTCCCCCCTCACC
TCCGGTGAGTCTGATCAGTGCCCTCGTCAGAGCCCACGTCGACTCCAATCC
GGCAATGACCAGCCTGGACTATTCCAGGTTCCAGGCAAACCCTGACTATC
AGATGAGTGGAGATGATACTCAACATATCCAGCAGTTCTACGATCTCCTG
ACTGGCTCTATGGAGATCATCAGAGGGTGGGCAGAGAAGATTCCTGGCTT
TGCTGACCTGCCCAAAGCCGATCAGGACCTGCTTTTTGAATCAGCTTTCTT
AGAATTATTTGTTCTACGCTTAGCATACAGGTCCAACCCAGTGGAGGGTA
AACTCATCTTTTGCAATGGGGTGGTCCTGCACAGGTTGCAATGCGTGCGTG
GCTTTGGGGAATGGATTGATTCCATTGTTGAATTCTCCTCCAACTTGCAGA
ATATGAACATCGACATTTCTGCCTTCTCCTGCATTGCTGCCCTGGCTATGG
TCACAGAGAGACACGGGCTCAAGGAACCCAAGAGAGTGGAAGAGCTACA
AAACAAAATTGTAAATTGTCTTAAAGACCATGTGACTTTCAATAATGGGG
GATTGAACCGACCCAACTACCTGTCCAAACTGTTGGGGAAGCTCCCAGAA
CTTCGCACCCTTTGCACACAGGGGCTCCAGCGCATTTTCTACCTGAAATTG
GAAGACTTGGTACCACCACCAGCAATAATTGACAAACTTTTCCTGGACAC
CTTACCTTTCTAAGACTTTCTCCCATGCACGTCTCGAGTACGAATCACTAG
TGAATTCGCGGCCGCCTGCAGGTCGAC

Appendix 3: Sequence of rat Nurr1. In red idicating the start codon, in blue the stop codon. Turquoise codon indicates silent mutation. Underlined is part of the primer sequence used for the PCR reaction. The sequence was also confirmed using reverse sequence primers but the result was not illustrated here.